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**TITLE:** Development of Novel PD1/PD-L1 Antagonists Using Circular Cys-Knotted Micro Proteins

**PRINCIPAL INVESTIGATOR:** Julio A. Camarero

**CONTRACTING ORGANIZATION:** University of Southern California  
Los Angeles, CA90089-9121

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14. ABSTRACT During this first year we have constructed genetically-encoded libraries using the loops 1 and 5 of cyclotide MCOTI-I. We have also accomplished the design and expression of a FRET-based reporter to screen antagonists for the PD-1/PD-L1 complex.						
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## 1. INTRODUCTION:

Carcinoma of the lung is one of the most common types of cancer worldwide. Lung cancer causes more deaths than the next three most common cancers combined (colon, breast and pancreatic). It is estimated that just in the United States around 160,000 people are expected to die from lung cancer in 2015, accounting for approximately 27% of all cancer deaths. This highlights the need for more effective therapies to treat this type of lethal disease. Increasing evidence shows that tumors can evade adaptive immunity and disrupt T-cell checkpoint pathways. The interaction between the PD-1 receptor and its ligands PD-L1 and PD-L2 is a key pathway hijacked by tumor cells to evade immune control. Hence, reversing the inhibition of the adaptive immunity can lead to the activation of a patient's immunity. For example, inhibition of the checkpoint pathways should block tumor immune inhibition. To date, several antagonistic mAbs have developed against the cytotoxic CTLA-4, the PD-1 receptor and PD-L1, to block immune checkpoints, and facilitate antitumor activity. These checkpoint-blocking antibodies have demonstrated clinical activity in a variety of tumor types, including melanoma, renal cell carcinoma, and lung cancer. Lung tumor cells have been found to express PD-L1 allowing the tumors to directly suppress anti-tumor cytolytic T cell activity and T cell down-regulation and inhibition. Blocking the interaction of the PD-1 receptor with one of its ligands, PD-L1, using mAbs has shown to increase the T cell response against the tumor. Two clinical trials involving the use of therapeutic mAbs able to block the PD-1/PD-L1 pathway have shown very promising results in lung cancer.

**Objectives.** Therapeutic mAbs are the fastest growing class of new therapeutic molecules. They hold great promises for the treatment of a variety of diseases, including cancer and chronic inflammatory diseases. However, the current manufacturing and purification processes cause limitations in the production capacity of therapeutic antibodies, leading to an increase in cost. We propose to use a micro-protein-based molecular scaffold (also cyclotide) for generating molecular libraries that will be screened and selected for potential antagonists for the PD-1/PD-L1 interaction. These compounds will be then screened and selected for their ability to antagonize the interaction between PD-1 and PD-L1 inside the bacterial cell using a genetically-encoded FRET-based reporter. We will use high throughput flow cytometry to identify bacteria encoding cyclotides able to specifically disrupt the soluble PD-1/PD-L1 complex. Selected cyclotides will be structurally characterized by NMR and assayed *in vitro* first to evaluate their ability to bind cells expressing PD-L1 and to antagonize the PD-1/PD-L1 pathway. Cyclotides with potent *in vitro* PD1/PD-L1 inhibitory properties will be further tested *in vivo* using immunocompetent syngeneic mouse models of lung cancer.

### Specific Aims

**Specific Aim 1.** To screen and select cyclotide-based peptides able to disrupt the PD-1/PD-L1 interaction. The objectives of this aim are 1) the production of large genetically-encoded libraries of cyclotides and 2) the production of cellular FRET-based screening

reporter to select cyclotides able to inhibit PD-1/PD-L1. Cells able to express active cyclotides will be selected using high throughput flow cytometry methods such as fluorescence activated cell sorting (FACS).

**Specific Aim 2.** To test and evaluate the inhibitory and biological activity of selected cyclotides *in vitro*. The objectives of this aim are 1) test selected cyclotides *in vitro* using a combination of fluorescence assays and nuclear magnetic resonance (NMR) and 2) evaluate their ability to block the PD1/PD-L1 pathway activity.

**Specific Aim 3.** To evaluate *in vivo* efficacy of the most potent cyclotide as a single agent. The objectives of this aim are to 1) evaluate *in vivo* efficacy and 2) toxicity of the most promising cyclotide. This will be accomplished using immunocompetent syngeneic mouse models of lung cancer, and bioactive cyclotides.

**2. KEYWORDS:**

Cyclotides, microproteins, immunotherapy, immune checkpoint, PD-1, PD-L1

### 3. ACCOMPLISHMENTS:

- **What were the major goals of the project?**
  - *Major Task 1. Develop a cell-based genetically encoded FRET-based reporter to screen antagonists for the PD-1/PD-L1 complex.*
  - *Major Task 2. Creation of cyclotide-based libraries using the MCoTI-cyclotide molecular scaffold in E. coli.*
  - *Major Task 3. Screen libraries of circular peptides inside E. coli cells to select potential cyclotides able to antagonize the PD-1/PD-L1 complex.*
  - *Major Task 4. In vitro characterization of selected cyclotides able to antagonize PD-1/PD-L1.*
  - *Major Task 5. Evaluate in biological activity of bioactive cyclotides in syngenic mouse models of lung cancer.*
- **What was accomplished under these goals?**
  - *Major Task 1. Develop a cell-based genetically encoded FRET-based reporter to screen antagonists for the PD-1/PD-L1 complex.* We have developed a FRET-reporter for the PD-1/PD-L1 interaction. We used the CyPet and YPet fluorescent proteins as a FRET-couple to monitor the interaction between PD-1 and PD-L1. To facilitate the interaction between targeted domains and prevent any steric hindrance that will interfere with the molecular recognition process, we used an appropriate flexible polypeptide linkers (i.e. [GGS]<sub>5</sub>) at the junctions between the interacting extracellular protein domains and the corresponding fluorescent proteins. Briefly, the extracellular domain of murine PD-L1 (residues 18-239, with a Met added to the N-terminus) and murine PD-1 (residues 25-157, with unpaired Cys83 mutated to Ser and a Met-Ala added to the N-terminus) were fused to C-terminal of CyPet and YPet, respectively. Please note the N-fusions resulted in poor yield expression. We have also developed a poly-cistronic expression plasmid for the co-expression of both fluorescent proteins, PD-1 and PD-L1, in E. coli cells to perform in-cell screening of inhibitors against the PD-1/PD-L1 complex.
  - *Major Task 2. Creation of cyclotide-based libraries using the MCoTI-cyclotide molecular scaffold in E. coli.* We have produced a genetically encoded library using the loops 1 and 5 of cyclotide MCoTI-I containing around 10 billion different sequences. This library was created at the DNA level using double stranded DNA inserts with degenerate sequences for loops 1 and 5 of cyclotide MCoTI-I. Briefly, a long degenerate synthetic oligonucleotide encoding the whole cyclotide, ≈100 nucleotide-long template is PCR amplified using 5'- and 3'-primers corresponding to the non-degenerate flanking regions. The resulting double-stranded degenerate DNA was double digested and then ligated to a linearized intein-encoding expression vector to produce a library of pASK-based plasmids. These libraries were then transformed into electrocompetent E. coli cells previously transformed with the FRET-based reporter to

finally obtain a library of cells typically containing up to  $\approx 10^9$  different clones (i.e. cyclotide sequences).

- ***Major Task 3. Screen libraries of circular peptides inside E. coli cells to select potential cyclotides able to antagonize the PD-1/PD-L1 complex.*** *This part of the work is currently being done and will be completed during the second year.*
- ***Major Task 4. In vitro characterization of selected cyclotides able to antagonize PD-1/PD-L1.*** *This part of the work will be completed during the second year.*
- ***Major Task 5. Evaluate in biological activity of bioactive cyclotides in syngenic mouse models of lung cancer.*** *This part of the work will be completed during the second year.*
- **What opportunities for training and professional development has the project provided?**
  - *Nothing to report.*
- **How were the results disseminated to communities of interest?**
  - *Some of the results/technologies developed in this proposal have been disseminated in conferences and peer-reviewed reviews.*
- **What do you plan to do during the next reporting period to accomplish the goals?**
  - *During the last year of the grant we will continue the screening of our cyclotide-based libraries. Selected hits will be further characterized biochemically for binding, and the most active compounds will be tested in animal models of lung cancer.*

4. **IMPACT:**

- **What was the impact on the development of the principal discipline(s) of the project?**
  - *We have developed for the first the production of genetically-encoded libraries of cyclotides. These libraries, containing billions of different microproteins, would provide a source of cyclotides to antagonize PD-1/PD-L1 complex but also other pharmacologically relevant cancer validated molecular targets.*
  - *The development of a FRET-based screening system to select PD-1/PD-L1 antagonist could be used also for in vitro high throughput screening of protein, peptides and small molecules.*
- **What was the impact on other disciplines?**
  - *Nothing to report.*
- **What was the impact on technology transfer?**
  - *Nothing to report.*
- **What was the impact on society beyond science and technology?**
  - *Nothing to report.*

5. **CHANGES/PROBLEMS:**

- **Changes in approach and reasons for change**
  - *Nothing to report.*
- **Actual or anticipated problems or delays and actions or plans to resolve them**
  - *We found some delays on the designing of the PD-1 and PD-L1 fluorescent based reporter due to initial problems with the solubility of these proteins when expressed in *E. coli*. This was solved by fusing the fluorescent protein to the N-terminal of the extracellular domains of PD-1 and PD-L1.*
- **Changes that had a significant impact on expenditures**
  - *Nothing to report.*
- **Significant changes in use or care of human subjects, vertebrate animals, biohazards, and/or select agents**
  - *Nothing to Report.*
- **Significant changes in use or care of human subjects**
  - *No human subjects were involved in this work.*
- **Significant changes in use or care of vertebrate animals.**
  - *Nothing to Report.*
- **Significant changes in use of biohazards and/or select agents**
  - *Nothing to Report.*

## 6. PRODUCTS:

- Publications, conference papers, and presentations
  - Journal publications.
    - 1) A. Gould and J. A. Camarero (2017) Cyclotides: Overview and biotechnological applications, *ChemBioChem*, doi: 10.1002/cbic.201700153. DoD-CDMRP acknowledged: Yes.
    - 2) K. Jagadish and J. A. Camarero (2017) Recombinant expression of cyclotides using split inteins, *Methods Mol. Biol.*, **1495**, 41-55. DoD-CDMRP acknowledged: No.
  - Oral Presentations
    - 1) Invited talk to Novartis: *Cyclotides, a new molecular scaffold to target protein-protein interactions*, May 3, 2017, Cambridge, Massachusetts. DoD-CDMRP acknowledged: Yes.
    - 2) Invited talk to the 13<sup>th</sup> Annual PEGS at Boston 2017 – Protein Engineering stream: *Rapid Screening of Cyclotide-Based Libraries against Intracellular Protein-Protein Interactions*, May 1, 2017, Boston, Massachusetts. DoD-CDMRP acknowledged: Yes.
    - 3) Invited talk to the Gordon Research Conference in Antimicrobial Peptides 2017: *Using Antimicrobial Cys-Rich Polypeptides as Scaffolds to Target Protein-Protein Interactions in Cancer*, March 1, 2017, Ventura, California. DoD-CDMRP acknowledged: Yes.
    - 4) Invited talk to the PepTalk 2017 meeting – The power of microbes to produce novel proteins and products: *Recombinant expression of circular Cys-knotted microproteins. Application for in-cell high throughput screening of specific protein-protein antagonists*, January 12, 2017, San Diego, California. DoD-CDMRP acknowledged: Yes.
    - 5) Invited talk to the Research Seminar Series at the Department of Medicinal Chemistry, School of Pharmacy, University of Michigan: *New Molecular Scaffolds to Target Protein-Protein Interactions*, September 15, 2016, Ann Arbor, Michigan. DoD-CDMRP acknowledged: Yes.
    - 6) Invited talk to Ipsen Biosciences: *Cyclotides, a new molecular scaffold to target protein-protein interactions*, August 26, 2016, Cambridge, Massachusetts. DoD-CDMRP acknowledged: Yes.
    - 7) Invited talk to the Children Hospital at Los Angeles (CHLA): *New molecular scaffold to target protein-protein interactions*, June 22, 2016, Los Angeles California. DoD-CDMRP acknowledged: Yes.
- Website(s) or other Internet site(s)
  - Nothing to report
- Technologies or techniques
  - 1) Developed new high throughput FRET-based assay to screen libraries of compounds in cell or in vitro. 2) Generated genetically-encoded libraries of cyclotide MCoT1-I using loops 1 and 5.

- **Inventions, patent applications, and/or licenses**
  - *Nothing to report yet.*
- **Other Products**
  - *Nothing to report.*

## 7. PARTICIPANTS & OTHER COLLABORATING ORGANIZATIONS

- What individuals have worked on the project?

Name:	<i>Julio A. Camarero</i>
Project Role:	<i>PI</i>
Researcher Identifier (e.g. ORCID ID):	<i>0000-0001-9150-0665</i>
Nearest person month worked:	<i>No change</i>
Contribution to Project:	<i>No change.</i>
Funding Support:	
Name:	<i>Nouri Neamati</i>
Project Role:	<i>coinvestigator</i>
Researcher Identifier (e.g. ORCID ID):	<i>0000-0003-3291-7131</i>
Nearest person month worked:	<i>No change</i>
Contribution to Project:	<i>No change.</i>
Funding Support:	
Name:	<i>Jagadish Krishnappa</i>
Project Role:	<i>Postdoc</i>
Researcher Identifier (e.g. ORCID ID):	
Nearest person month worked:	<i>12 months</i>
Contribution to Project:	<i>Left research group on June 1<sup>st</sup>, he won't be contributing to the 2<sup>nd</sup> year, his role would be replaced by Dr. Corina Sarmiento.</i>
Funding Support:	
Name:	<i>Teshome Aboye</i>
Project Role:	<i>Postdoc</i>
Researcher Identifier (e.g. ORCID ID):	
Nearest person month worked:	<i>No change</i>

Contribution to Project:	<i>No change.</i>
Funding Support:	
Name:	<i>Corina Sarmiento</i>
Project Role:	<i>Postdoc (to replace Dr. Jagadish Krishnappa)</i>
Researcher Identifier (e.g. ORCID ID):	
Nearest person month worked:	<i>1 month</i>
Contribution to Project:	<i>1 month in first year. In the 2<sup>nd</sup> year she will contribute full time (100%)</i>
Funding Support:	

- **Has there been a change in the active other support of the PD/PI(s) or senior/key personnel since the last reporting period?**
  - *Nothing to Report.*
- **What other organizations were involved as partners?**
  - *Nothing to Report.*

**8. SPECIAL REPORTING REQUIREMENTS**

- **COLLABORATIVE AWARDS:** *No required..*
- **QUAD CHARTS:** *No required.*

9. **APPENDICES:** *Attach all appendices that contain information that supplements, clarifies or supports the text. Examples include original copies of journal articles, reprints of manuscripts and abstracts, a curriculum vitae, patent applications, study questionnaires, and surveys, etc. Reminder: Pages shall be consecutively numbered throughout the report. DO NOT RENUMBER PAGES IN THE APPENDICES.*

# Chapter 4

## Recombinant Expression of Cyclotides Using Split Inteins

Krishnappa Jagadish and Julio A. Camarero

### Abstract

Cyclotides are fascinating microproteins ( $\approx 30$  residues long) present in several families of plants that share a unique head-to-tail circular knotted topology of three disulfide bridges, with one disulfide penetrating through a macrocycle formed by the two other disulfides and inter-connecting peptide backbones, forming what is called a cystine knot topology. Naturally occurring cyclotides have shown to possess various pharmacologically relevant activities and have been reported to cross cell membranes. Altogether, these features make the cyclotide scaffold an excellent molecular framework for the design of novel peptide-based therapeutics, making them ideal substrates for molecular grafting of biological peptide epitopes. In this chapter we describe how to express a native folded cyclotide using intein-mediated protein trans-splicing in live *Escherichia coli* cells.

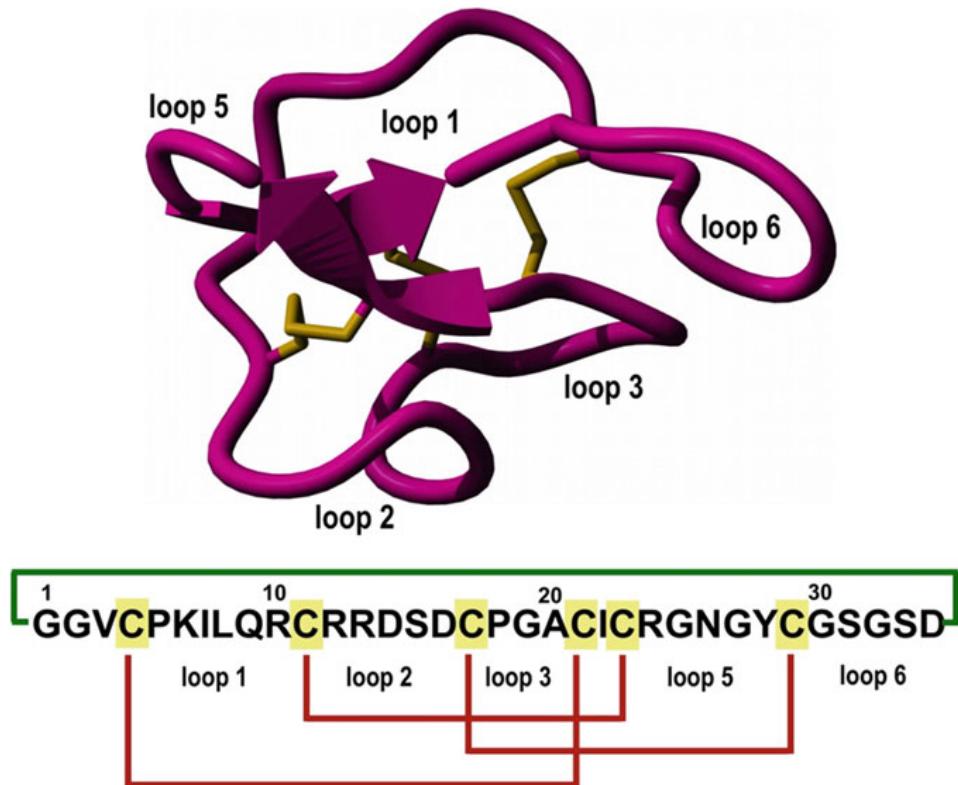
**Key words** Cyclotides, CCK motif, Split-intein, Protein trans-splicing, *Npu* intein

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### 1 Introduction

Cyclotides are small globular microproteins (ranging from 28 to 37 residues) containing a unique head-to-tail cyclized backbone topology that is stabilized by three disulfide bonds to form a cystine-knot (CCK) motif [1, 2] (Fig. 1). This CCK molecular framework provides a very rigid molecular platform [3–5] conferring an exceptional stability towards physical, chemical, and biological degradation [1, 2]. In fact, the use of cyclotide-containing plants in indigenous medicine first highlighted the fact that the peptides are resistant to boiling and are orally bioavailable [6].

Cyclotides can be considered as natural combinatorial peptide libraries structurally constrained by the cystine-knot scaffold and head-to-tail cyclization but in which hypermutation of essentially all residues is permitted with the exception of the strictly conserved cysteines that comprise the knot [7–9]. The main features of cyclotides are a remarkable stability due to the cystine knot, a small size making them readily accessible to chemical synthesis, and an excellent tolerance to sequence variations. Naturally occurring



**Fig. 1** Tertiary structure of the cyclotide MCoTI-II (PDB code: 1IB9) and primary structures of cyclotides used in this study. The backbone cyclized peptide (connecting bond shown in green) is stabilized by the three disulfide bonds (shown in red)

cyclotides have shown to possess various pharmacologically relevant activities [1, 10]. Cyclotides have been also engineered to target extracellular [11–13] and intracellular [14] molecular targets in animal models. Some of these novel cyclotides are orally bioavailable [12] and are able to cross cellular membranes efficiently [15, 16]. Cyclotides thus appear as highly promising leads or frameworks for peptide drug design [10, 17].

Naturally occurring cyclotides are ribosomally produced in plants from precursors that comprise between one and three cyclotide domains [18–21]. However, the mechanism of excision of the cyclotide domains and ligation of the free N- and C-termini to produce the circular peptides has not yet been completely elucidated although it has been speculated that asparaginyl endopeptidases are involved in the cyclization process [22–24]. Cyclotides can be also produced recombinantly using standard microbial expression systems by making use of modified protein splicing units [25–28] allowing for the first time the production of biologically generated libraries of these microproteins [26].

We describe in this chapter how to produce cyclotide MCoTI-I (Fig. 1) in *E. coli* cells making use of protein trans-splicing. Cyclotide MCoTI-I is a very potent trypsin inhibitor ( $K_i \approx 20$  pM) [27] that has been recently isolated from dormant seeds of

*Momordica cochinchinensis*, a plant member of the *Cucurbitaceae* family [29]. Trypsin inhibitor cyclotides are interesting candidates for drug design because they can cross mammalian cell membranes [15, 16] and their specificity for inhibition can be altered and their structures can be used as natural scaffolds to generate novel binding activities [11, 14]. Protein trans-splicing is a post-translational modification similar to protein splicing with the difference that the intein self-processing domain is split into N- ( $I_N$ ) and C-intein ( $I_C$ ) fragments. The split-intein fragments are not active individually; however, they can bind to each other with high specificity under appropriate conditions to form an active protein splicing or intein domain in *trans* [30]. PTS-mediated backbone cyclization can be accomplished by rearranging the order of the intein fragments. By fusing the  $I_N$  and  $I_C$  fragments to the C- and N-termini of the polypeptide for cyclization, the trans-splicing reaction yields a backbone-cyclized polypeptide (Fig. 2).

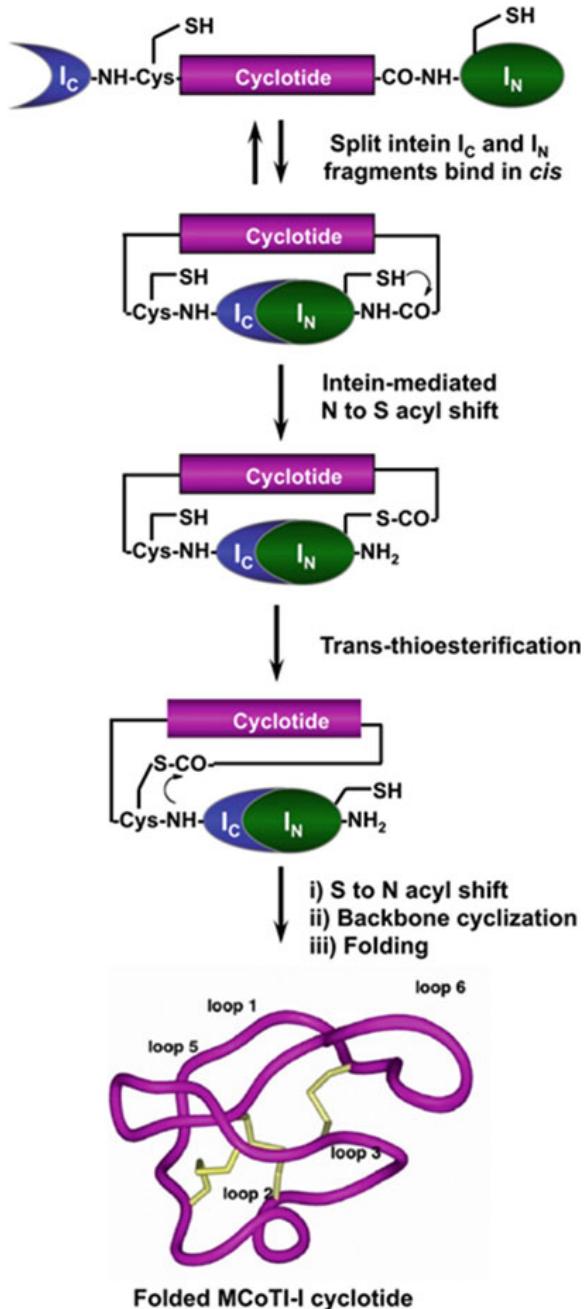
In cell cyclization and folding of cyclotide MCoTI-I will be accomplished using the naturally occurring *Nostoc punctiforme* PCC73102 (*Npu*) DnaE split-intein. This DnaE intein has the highest reported rate of protein trans-splicing ( $\tau_{1/2} \approx 60$  s) [31], high splicing yield [31, 32] and has shown high tolerance to the amino acid composition of the intein–extein junctions for efficient protein splicing [25, 33]. To accomplish this, we designed the split-intein construct 1 (Fig. 3). In this construct, the MCoTI-I linear precursor was fused in-frame at the C- and N-termini directly to the *Npu* DnaE  $I_N$  and  $I_C$  polypeptides. None of the additional native C- or N-extein residues were added in this construct. We used the native Cys residue located at the beginning of loop 6 of MCoTI-I (Fig. 1) to facilitate backbone cyclization. A His-tag was also added at the N-terminus of the construct to facilitate purification. In-cell expression of cyclotide MCoTI-I using PTS-mediated backbone cyclization was achieved by transforming the plasmid encoding the split-precursor 1 into Origami 2(DE3) cells to facilitate folding.

## 2 Materials

All solutions were prepared using ultrapure water with a resistivity of  $18 \text{ M}\Omega \times \text{cm}$  at  $25^\circ\text{C}$  and analytical grade reagents. All reagents and solutions were stored at room temperature unless indicated otherwise.

### 2.1 Instruments

1. Sonicator for cell lysis (e.g., Sonifier 250 Branson CA, USA).
2. 5 mL polypropylene columns (QIAGEN).
3. Lyophilizer (e.g., Flexi Dry™ μp, Frigeco Inc. USA).
4. HPLC system equipped with gradient capability and UV–Vis detection (e.g., Agilent 1100, Agilent Technologies, USA).



**Fig. 2** In-cell expression of native folded cyclotide MCoTI-I using intein-mediated protein trans-splicing



**MCoTI-intein construct, 1**

**Fig. 3** Architecture of the intein precursor used for the expression of cyclotide MCoTI-I described in this protocol

5. C18 reverse phase HPLC columns (e.g., Vydac C18 column 5  $\mu$ m, 4.6  $\times$  150 mm and 2.1  $\times$  100 mm columns, Grace Discovery Sciences, USA).
6. Electrospray mass spectrometer (ES-MS) (e.g., API-3000, Applied Biosystems, USA).

## 2.2 Cloning of MCoTI-Intein Construct 1

1. DNA ultramers encoding MCoTI-I, *Npu* DnaE  $I_C$  and  $I_N$  were ordered as synthetic oligonucleotides (20 nmol scale and purified by PAGE) (Table 1).
2. TE buffer: 10 mM Tris-HCl, 1 mM EDTA, pH 8.0.

**Table 1**

ssDNA sequences used to generated the dsDNA fragments encoding *Npu* DnaE  $I_C$  and  $I_N$ , and MCoTI-I

Name of Sequence	Nucleotide sequence
P5- $I_C$	C ATG GGC AGC AGC CAT CAT CAT CAT CAC AGC AGC GGC CTG GTG CCG CGC GGC AGC ATG ATC AAA ATA GCC ACA CGT AAA TAT TTA GGC AAA CAA AAT GTC TAT GAC ATT GGA GTT GAG CGC GAC CAT AAT TTT GCA CTC AAA AAT GGC TTC ATA GCT T
P3- $I_C$	CGA AGC TAT GAA GCC ATT TTT GAG TGC AAA ATT ATG GTC GCG CTC AAC TCC AAT GTC ATA GAC ATT TTG TTT GCC TAA ATA TTT ACG TGT GGC TAT TTT GAT CAT GCT GCC GCG CGG CAC CAG GCC GCT GCT GTG ATG ATG ATG ATG GCT GCT GCC C
P5-MCoTI	CGA ACT GCG GTT CTG GTT CTG ACG GTG GTG TTT GCC CGA AAA TCC TGC AGC GTT GCC GTC GTG ACT CTG ACT GCC CGG GTG CTT GCA TCT GCC GTG GTA ACG GTT ACT GTT TAT CA
P3-MCoTI	TAT GAT AAA CAG TAA CCG TTA CCA CGG CAG ATG CAA GCA CCC GGG CAG TCA GAG TCA CGA CGG CAA CGC TGC AGG ATT TTC GGG CAA ACA CCA CCG TCA GAA CCA GAA CCG CAG TT
P5- $I_N$	TAT GAA ACG GAA ATA TTG ACA GTA GAA TAT GGA TTA TTA CCG ATT GGT AAA ATT GTA GAA AAG CGC ATC GAA TGT ACT GTT TAT AGC GTT GAT AAT AAT GGA AAT ATT TAT ACA CAA CCT GTA GCA CAA TGG CAC GAT CGC GGA GAA CAA GAG GTG TTT GAG TAT TGT TTG GAA GAT GGT TCA TTG ATT CGG GCA ACA AAA GAC CAT AAG TTT ATG ACT GTT GAT GGT CAA ATG TTG CCA ATT GAT GAA ATA TTT GAA CGT GAA TTG GAT TTG ATG CGG GTT GAT ATT TTG CCG AAT TA
P3- $I_N$	AGC TTA ATT CGG CAA ATT ATC AAC CCG CAT CAA ATC CAA TTC ACG TTC AAA TAT TTC ATC AAT TGG CAA CAT TTG ACC ATC AAC AGT CAT AAA CTT ATG GTC TTT TGT TGC CCG AAT CAA TGA ACC ATC TTC CAA ACA ATA CTC AAA CAC CTC TTG TTC TCC GCG ATC GTG CCA TTG TGC TAC AGG TTG TGT ATA AAT ATT TCC ATT ATT ATC AAC GCT ATA AAC AGTA CAT TCG ATG CGC TTT TCT ACA ATT TTA CCA ATC GGT AAT AAT CCA TAT TCT ACT GTC AAT ATT TCC GTT TCA

DNA sequences were generated using optimal codons for expression in *E. coli*. Bases in red are as overhangs to facilitate ligation

- Annealing buffer: phosphate buffer, 20 mM Na<sub>2</sub>HPO<sub>4</sub>, 300 mM NaCl buffer, pH 7.4.
- QIAquick PCR Purification Kit (QIAGEN).
- QIAprep Spin Miniprep Kit (QIAGEN).
- QIAquick Gel Extraction Kit (QIAGEN).
- Synthetic DNA primers used to amplify DNA encoding MCoTI-intein construct 1 (20 nmol scale, HPLC purified) (Table 2).
- Vent DNA polymerase, TaqDNA polymerase, dNTPs solution, 10× ThermoPol PCR buffer, 10× TaqDNA polymerase buffer.
- Restriction enzymes: *NcoI* and *HindIII*.
- NEB buffer 2.1, 50 mM NaCl, 10 mM Tris-HCl, 10 mM MgCl<sub>2</sub>, 100 µg/mL bovine serum albumin (BSA), pH 7.9.
- Chemical competent DH5 $\alpha$  cells.
- Expression plasmid pET28a (Novagen-EMD Millipore).
- T4 DNA ligase buffer.
- LB medium: 25 g of LB broth was dissolved in 1 L of pure H<sub>2</sub>O and sterilized by autoclaving at 120 °C for 30 min.
- LB medium-agar: 3.3 g of LB agar was suspended in 100 mL of pure H<sub>2</sub>O and sterilized by autoclaving at 120 °C for 30 min. To prepare plates, allow LB medium-agar to cool to  $\approx$ 50 °C, then add 0.1 mL of kanamycin stock solution (25 mg kanamycin/mL in H<sub>2</sub>O, sterilized by filtration over a 45 µm filter), gently mix and pipet 20 mL into a sterile petri dish (100 mm diameter).
- SOC Medium: 20 g of tryptone, 5 g yeast extract, 0.5 g NaCl, and 0.186 g KCl was suspended into 980 mL of pure water and sterilized by autoclaving at 120 °C for 30 min. Dissolve 4.8 g MgSO<sub>4</sub>, 3.603 g dextrose in 20 mL of pure H<sub>2</sub>O and filter sterilize over a 45 µm filter and add to the autoclaved medium.

**2.3 Cyclotide Expression, Purification, and Characterization**

- Chemical competent Origami2 (DE3) cells (EMD Millipore).
- Isopropyl-thio- $\beta$ -D-galactopyranoside (IPTG), analytical grade. Prepare a stock solution of 1 M in H<sub>2</sub>O and sterilize by filtration over 45 µm filter. Store at -20 °C.

**Table 2**  
**DNA oligonucleotides used to generate the dsDNA encoding the MCoTI-intein precursor construct 1**

Name of Sequence	Nucleotide sequence
Forward I <sub>C</sub> primer	5'- AAA ACC ATG GGC AGC AGC CAT CAT CAT -3'
Reverse I <sub>N</sub> primer	5'- TTT TAA GCT TAA TTC GGC AAA TTA TCA ACC C -3'

3. Sterile conical bottom flasks.
4. Ni-lysis buffer: 10 mM imidazole, 50 mM Na<sub>2</sub>HPO<sub>4</sub>, 150 mM NaCl, pH 8.
5. Ni-wash buffer: 20 mM imidazole, 50 mM Na<sub>2</sub>HPO<sub>4</sub>, 150 mM NaCl, pH 8.
6. Ni-elution buffer: 50 mM Na<sub>2</sub>HPO<sub>4</sub>, 150 mM NaCl, 250 mM imidazole, pH 8.
7. Ni-NTA agarose beads (EMD MilliPore).
8. 100 mM phenylmethylsulfonyl fluoride (PMSF) in EtOH (better to prepare fresh before use).
9. 4× SDS-PAGE sample buffer: 1.5 mL of 1 M Tris-HCl buffer, pH 6.8, 3 mL of 1 M DTT (dithiothreitol) in pure H<sub>2</sub>O, 0.6 g of sodium dodecyl sulfate (SDS), 30 mg of bromophenol blue, 2.4 mL of glycerol, bring final volume to 7.5 mL.
10. SDS-PAGE sample buffer: dilute four times 4× SDS-PAGE sample buffer in pure H<sub>2</sub>O and add 20% 2-mercaptoethanol (by volume). Prepare fresh.
11. SDS-4–20% PAGE gels, 1× SDS running buffer.
12. Gel stain: Gelcode® Blue (Thermo scientific).
13. N-hydroxy-succinimide ester (NHS)-activated sepharose beads (GE Healthcare life sciences).
14. Porcine pancreatic trypsin type Ix-S (14,000 units/mg) (Sigma Aldrich).
15. Coupling buffer: 200 mM sodium phosphate, 250 mM NaCl, pH 6.0.
16. Washing buffer: 200 mM sodium acetate, 250 mM NaCl, pH 4.5.
17. Column buffer: 0.1 mM ethylenediaminetetraacetic acid (EDTA), 50 mM Na<sub>2</sub>HPO<sub>4</sub>, 150 mM NaCl, pH 7.4.
18. 100 mM ethanolamine (Eastman Kodak).
19. 8 M guanidinium chloride in pure water for molecular biology.
20. Solid-phase extraction silica-C18 cartridge (820 mg of silica-C18, 55–105 µm particle size) (Sep-Pak C18 Plus Long Cartridge, Waters).
21. HPLC buffers. Buffer A: pure and filtered (over 45 µm filter) H<sub>2</sub>O with 0.1% trifluoroacetic acid (TFA) (HPLC grade). Buffer B: 90% acetonitrile (HPLC grade) in pure and filtered (over 45 µm filter) H<sub>2</sub>O with 0.1% TFA.

### 3 Methods

#### 3.1 Construction of Intein-MCoTI-I

##### Construct 1

###### 3.1.1 Annealing of the DNA Fragments

Encoding Split Intein *Npu DnaE* and Cyclotide MCoTI-I

1. Dissolve each ultramer shown in Table 1 in TE buffer to a concentration of 1  $\mu$ g/ $\mu$ L with TE buffer.
2. The annealing reaction for every DNA fragment (*Npu DnaE I<sub>C</sub>* and *I<sub>N</sub>*, and MCoTI-I) is carried out as follows: 5  $\mu$ L of solution containing the upper DNA strand (P5) and 5  $\mu$ L of solution containing the lower strand (P3) are added into a 0.5 mL centrifuge tube containing 2.5  $\mu$ L of 10 $\times$  annealing buffer and 12  $\mu$ L of pure H<sub>2</sub>O. This should provide three annealing reactions for the DNA encoding regions corresponding to the *DnaE I<sub>N</sub>* and *I<sub>C</sub>*, and MCoTI-I polypeptides.
3. Incubate the above samples on a preheated water bath to 95 °C for 15 min. Turn off the power of the water bath, and allow the samples to slowly cool down to room temperature. The cooling process should not take less than 60 min.
4. Purify the double strand DNA fragments by using the QIAGEN PCR cleanup kit following the manufacturer instructions.
5. Double strand DNA fragments were obtained in TE buffer and quantified by UV-Vis spectroscopy (for a 1-cm path-length, an optical density at 260 nm (OD<sub>260</sub>) of 1.0 equals to a concentration of 50  $\mu$ g/mL solution of dsDNA).

###### 3.1.2 Ligation and Amplification of the DNA Fragment Encoding Construct 1

1. Mix equimolar amounts ( $\approx$ 20 nmol) of each dsDNA fragment encoding for the *DnaE I<sub>C</sub>/I<sub>N</sub>* and MCoTI-I polypeptides in a thin walled 0.5 mL centrifuge tube. Add enough pure sterile water to have a final volume reaction of 50  $\mu$ L, add 5  $\mu$ L of 10 $\times$  T4 DNA ligase buffer, 1  $\mu$ L of 10 mM ATP, 1  $\mu$ L of 10 mM dNTP, and then add 1  $\mu$ L (400 units) of T4 DNA ligase enzyme. Incubate the ligation reaction at 16 °C overnight.
2. Purify the ligated DNA encoding construct 1 using Qiagen PCR cleanup kit according to the manufacturer instructions, and quantify it by UV-Vis spectroscopy.
3. Amplify the construct by PCR using primers shown in Table 2, which introduce *NcoI* and *HindIII* restriction sites in the 5' and 3' positions of the coding DNA sequence. Carry out the PCR reaction as follows: 40  $\mu$ L sterile pure H<sub>2</sub>O, 1  $\mu$ L of ligated dsDNA ( $\approx$ 10 ng/ $\mu$ L), 5  $\mu$ L of 10 $\times$  ThermoPol reaction buffer, 1.0  $\mu$ L of dNTP solution (10 mM each), 1  $\mu$ L of forward *I<sub>C</sub>* primer solution (0.2  $\mu$ M), 1  $\mu$ L of *I<sub>N</sub>* reverse primer solution (0.2  $\mu$ M), and 1  $\mu$ L Vent DNA polymerase (2 units).
4. PCR cycle conditions used: initial denaturation at 94 °C for 5 min followed by 30 cycles (94 °C denaturation for 30 s, annealing at 56 °C for 45 s, and extension at 72 °C for 60 s) and final extension at 72 °C for 10 min.

5. Purify the PCR amplified fragment encoding construct 1 using the QIAquick PCR purification kit following the manufacturer instructions and quantify it by UV-visible spectroscopy.

*3.1.3 Preparation of Expression Plasmid pET28-MCoTI-TS*

1. Digest the plasmid pET28a (Novagen-EMD Millipore) and the PCR-amplified gene encoding MCoTI-intein construct with restriction enzymes *NcoI* and *HindIII*. Use a 0.5 mL centrifuge tube and add 5  $\mu$ L of NEB buffer 2.1 (New England Biolabs), add enough pure sterile water to have a final volume reaction of 50  $\mu$ L, add  $\approx$ 10  $\mu$ g of the corresponding dsDNA to be digested add finally add 1  $\mu$ L (20 units) of restriction enzyme *NcoI* (New England Biolabs). Incubate at 37 °C for 3 h. Then, add 1  $\mu$ L (20 units) of restriction enzyme *HindIII* (New England Biolabs) to the same tube and incubate at 37 °C for 1 h.
2. Purify the double digested PCR-product and pET28a plasmid by agarose (0.8 % and 2 % agarose gels for pET28s and PCR product should be used, respectively) gel electrophoresis. The bands corresponding to the double digested DNA are cut and purified using the QIAquick Gel Extraction Kit (QIAGEN), eluted with TE buffer and quantified using UV-visible spectroscopy.
3. Ligate double digested pET28a and PCR-product encoding MCoTI-intein construct 1. Use a 0.5 mL centrifuge tube, add  $\approx$ 100 ng of *NcoI*, *HindIII*-digested pET28a,  $\approx$ 50 ng of *NcoI*, *HindIII*-digested PCR-amplified DNA encoding MCoTI-intein construct 1, enough pure sterile H<sub>2</sub>O to make a final reaction volume of 20  $\mu$ L, 2  $\mu$ L of 10 $\times$  T4 DNA ligase buffer, 1  $\mu$ L of 10 mM ATP, and 1  $\mu$ L (400 units) T4 DNA ligase. Incubate at 16 °C overnight.
4. Transform the ligation mixture into DH5 $\alpha$  competent cells.  $\approx$ 100  $\mu$ L of chemical competent cells are thawed on ice and mixed with the ligation mixture (20  $\mu$ L) for 30 min. The cells are heat-shocked at 42 °C for 45 s and then kept on ice for an extra 10 min. Add 900  $\mu$ L of SOC medium and incubate at 37 °C for 1 h in an orbital shaker. Plate 100  $\mu$ L on LB agar plate containing kanamycin (25  $\mu$ g/mL) and incubate the plate at 37 °C overnight.
5. Pick up several colonies (most of the times five colonies should be enough) and inoculate into 5 mL of LB medium, 25  $\mu$ g/mL kanamycin. Incubate tubes at 37 °C overnight in an orbital shaker.
6. Pellet down cells and extract DNA using the QIAprep Spin Miniprep Kit (QIAGEN) following the manufacturer protocol and quantify plasmid using UV-visible spectroscopy.
7. Verify the presence of DNA encoding MCoTI-intein construct in each colony using PCR. Carry out the PCR reaction as follows: 40  $\mu$ L sterile pure H<sub>2</sub>O, 1  $\mu$ L of plasmid DNA ( $\approx$ 50 ng/ $\mu$ L), 5  $\mu$ L of 10 $\times$  TaqDNA polymerase buffer, 1.0  $\mu$ L of dNTP

solution (10 mM each), 1  $\mu$ L of forward  $I_C$  primer solution (0.2  $\mu$ M), 1  $\mu$ L of  $I_N$  reverse primer solution (0.2  $\mu$ M), and 1  $\mu$ L Taq DNA polymerase (5 units).

8. PCR cycle conditions used: initial denaturation at 94  $^{\circ}$ C for 5 min followed by 30 cycles (94  $^{\circ}$ C denaturation for 30 s, annealing at 56  $^{\circ}$ C for 45 s, and extension at 72  $^{\circ}$ C for 60 s) and final extension at 72  $^{\circ}$ C for 10 min.

### 3.2 Expression and Purification of Cyclotides

#### 3.2.1 Expression of Precursor Protein Encoding the MCoTI-Intein Construct 1

1. Transform chemical competent Origami2(DE3) cells with plasmid containing the DNA encoding MCoTI-intein construct 1 (plasmid pET28-MCoTI-TS) (see Note 1). Plate transformed cells on LB plate, 2  $\mu$ g/mL kanamycin, and incubate at 37  $^{\circ}$ C overnight as described in Subheading 3.1.3 (see Note 2).
2. Resuspend the colonies from two plates in 2 mL of LB medium and use resuspension to inoculate 1 L of LB medium, 25  $\mu$ g/mL kanamycin, in a 2.5 L flask.
3. Grow cells in an orbital shaker incubator at 37  $^{\circ}$ C for 2–3 h to reach mid-log phase (OD at 600 nm  $\approx$  0.5). Add IPTG to reach a final concentration of 0.3 mM. Adjust the temperature of the incubator to 25  $^{\circ}$ C and incubate cells in shaker for 16 h.
4. Pellet cells by centrifugation at  $6000 \times g$  for 15 min at 4  $^{\circ}$ C. Discard the supernatant and process the pellet immediately (see Note 3).

#### 3.2.2 Protein Extraction

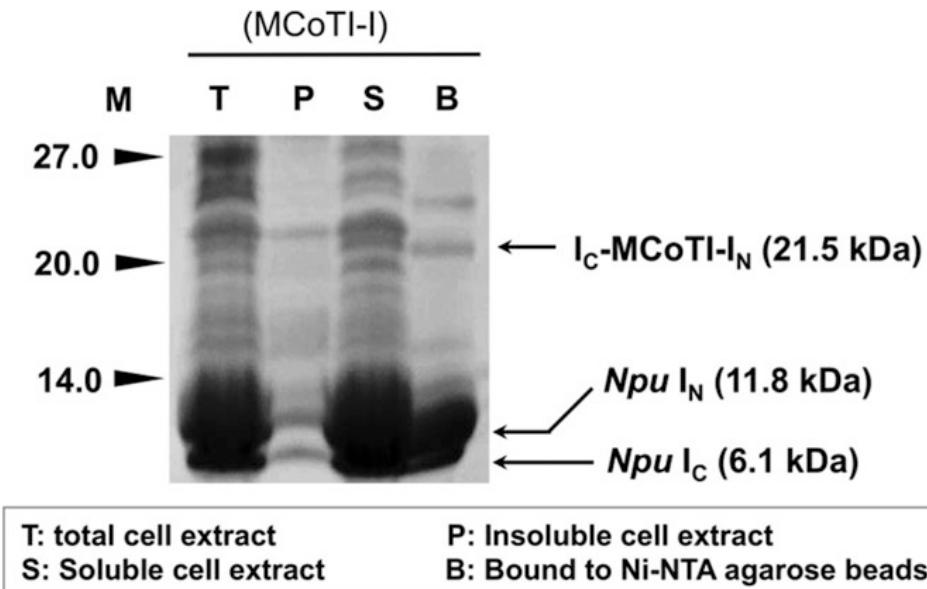
1. Resuspend cell pellet with 30 mL of Ni-lysis buffer containing 1 mM PMSF. Lyse cells by sonication on ice using 25 s bursts spaced 30 s each (see Note 4). Repeat the cycle six times (see Note 5). Take two 100  $\mu$ L aliquots. In one of the samples add 33  $\mu$ L 4 $\times$  SDS-PAGE sample buffer, 20% 2-mercaptoethanol, and heat it at 94  $^{\circ}$ C for 5 min (label it total cell lysate, T). For the other aliquot, separate the insoluble and soluble fractions by centrifugation at  $15,000 \times g$  in a microcentrifuge at 4  $^{\circ}$ C for 30 min. Take the supernatant fraction and resuspend the pellet in 100  $\mu$ L of Ni-lysis buffer. Add 33  $\mu$ L of 4 $\times$  SDS-PAGE sample buffer containing 20% 2-mercaptoethanol to both fractions and heat them at 94  $^{\circ}$ C for 5 min (label them soluble and insoluble cell lysate samples, P and S, respectively). Save the samples for later SDS-PAGE analysis.
2. Separate the soluble cell lysate fraction by centrifugation at  $15,000 \times g$  for 20 min at 4  $^{\circ}$ C. Stored the pellets at  $-80$   $^{\circ}$ C in case they need to be reprocessed.
3. Transfer the soluble cell lysate fraction ( $\approx$ 30 mL) into a 50 mL centrifuge tube and add 1 mL of pre-equilibrated Ni-NTA-agarose beads. Incubate with gentle rocking for 30 min at 4  $^{\circ}$ C.
4. Separate the beads from supernatant by centrifugation at  $3000 \times g$  for 10 min at 4  $^{\circ}$ C. Take the supernatant and save it at 4  $^{\circ}$ C for

later analysis (Subheading 3.2.4). Separate the beads and wash them in a 5 mL polypropylene column with no less than 15 column volumes of Ni-wash buffer. Take  $\approx$ 100  $\mu$ L of Ni-NTA-agarose beads into a 0.5 mL centrifuge tube, add 33  $\mu$ L of 4 $\times$  SDS-PAGE sample buffer, 20% 2-mercaptoethanol, and heat it at 94 °C for 5 min (label it soluble cell lysate bound to Ni-NTA agarose beads, B). Save the sample for later SDS-PAGE analysis.

5. Analyze the expression level of the precursor protein 1 using SDS-PAGE (Fig. 4). Load 25  $\mu$ L of samples labeled T, P, S, and B (see above) onto an SDS-4–20% PAGE gel. Run the samples at 125 V for about 1 h and 30 min in 1 $\times$  SDS running buffer. Remove SDS with pure water and stain the gel with 20 mL GelCode® Blue reagent (see Note 6) using the manufacturer protocol (Fig. 4).

### 3.2.3 Preparation of Trypsin-Immobilized Agarose Beads for Affinity Chromatography

1. Wash  $\approx$ 1 mL of NHS-activated sepharose with 15 column volumes of ice-cold 1 mM HCl using a 5 mL polypropylene column.
2. Equilibrate column with 15 volumes of coupling buffer.
3. Dissolve 4 mg of porcine pancreatic trypsin in 500  $\mu$ L of coupling buffer using gentle rocking.
4. Add the trypsin solution to the equilibrated NHS-activated sepharose beads and incubate for 3 h with gentle rocking at room temperature.

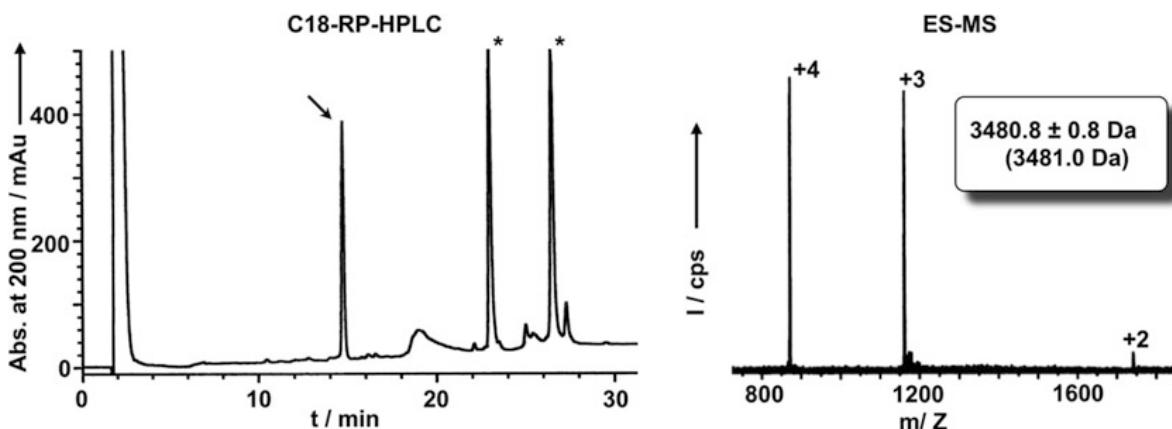


**Fig. 4** SDS-PAGE analysis of the recombinant expression of cyclotide precursors **1** in Origami2(DE3) cells for in-cell production of cyclotide MCoTI-I. The bands corresponding to precursor 1, and  $I_N$  and  $I_C$  polypeptides are marked with arrows. Despite that only the  $I_C$  polypeptide has a His-tag, the  $I_N$  binds the  $I_C$  polypeptide with low nM affinity and is co-purified during the Ni-NTA pre-purification step. M, stands for protein markers

5. Wash the sepharose beads with ten volumes of coupling buffer containing 100 mM ethanolamine.
6. Incubate beads with three column volumes of coupling buffer containing 100 mM ethanolamine for 3 h with gentle rocking at room temperature.
7. Wash the sepharose beads with 50 column volumes of washing buffer and store a 4 °C until use (see Note 7).

*3.2.4 Affinity-Purification of Cyclotide MCoTI-I from Bacterial Soluble Cell Lysate*

1. Wash  $\approx$ 500  $\mu$ L of trypsin-sepharose beads with ten column volumes of column buffer.
2. Incubate the washed beads with the soluble cell lysate flow-through from the Ni-NTA agarose beads purification step (Subheading 3.2.2, step 4) for 1 h at room temperature with gentle rocking.
3. Separate the supernatant by centrifugation at  $3000 \times g$  for 10 min at 4 °C.
4. Transfer the trypsin-beads to a 5 mL polypropylene column and wash the beads with 50 volumes of column buffer containing 0.1% Tween 20.
5. Wash trypsin-beads with 50 volumes of column buffer with no detergent added.
6. Elute bound cyclotide MCoTI-I with three volumes ( $3 \times 500 \mu$ L) of 8 M guanidinium hydrochloride at room temperature for 15 min by gravity.
7. Desalt the sample using a solid-phase extraction cartridge (SepPak, Waters) by following the manufacturer protocol. Briefly, pre-swell the SepPak cartridge with 20 mL of 50% acetonitrile in water containing 0.1% TFA. Equilibrate cartridge with 50 mL of HPLC buffer A. Load sample into cartridge using a plastic 20 mL syringe slowly with a flow rate of  $\approx$ 1 mL/min. Collect flow-through and repeat this step for efficient binding. Wash the cartridge with 20 mL of 5% acetonitrile in H<sub>2</sub>O containing 0.1% TFA. Elute cyclotide MCoTI-I from the solid-phase extraction cartridge with 5 mL of HPLC buffer-B. Lyophilize to remove solvents.
8. Dissolve in 5 mL of HPLC buffer A. Analyze sample by HPLC using an isocratic of 0% buffer B for 2 min and then a linear gradient of 0–70% buffer B in 30 min. Use detection at 220 and 280 nm. Using these conditions the retention time of the cyclotide should be around 15 min (Fig. 5). Collect the peak and analyze by mass spectrometry to confirm identity of cyclotide MCoTI-I (Fig. 5; expected molecular weight: 3481.0 Da).
9. Quantify the cyclotide using UV-visible spectroscopy and a molar absorptivity at 280 nm of  $2240 \text{ M}^{-1} \times \text{cm}^{-1}$ . Around 150  $\mu$ g of folded cyclotide should be obtained per liter of LB culture.



**Fig. 5** Analytical HPLC trace (*left panel*) of the soluble cell extract of bacterial cells expressing precursor **1** after purification by affinity chromatography on a trypsin-sepharose column. Folded MCoTI-I is marked with an arrow. Endogenous bacterial proteins that bind trypsin are marked with an asterisk. Mass spectrum (*right panel*) of affinity purified MCoTI-I. The expected average molecular weight is shown in parentheses

#### 4 Notes

1. We recommend to screen at least 3–4 different colonies for protein expression to make sure that the MCoTI-I intein construct **1** is expressed efficiently.
2. When plating the transformed cells with pET28-MCoTI-TS, it is better to aim for plates containing 200–300 colonies.
3. Cell pellets can be stored at  $-80^{\circ}\text{C}$  for no more than 2–3 weeks before being processed.
4. During sonication, be sure the temperature of the sample does not overheat.
5. A french press can be also used to lyse cells, depending on the availability.
6. Coomassie brilliant blue can be also used for staining PAGE gels.
7. The trypsin-sepharose column should not be stored for more than 2 weeks. The loading of the trypsin-sepharose can be determined by incubating a small aliquot of the beads with a known amount of pure MCoTI-I and determining the amount of cyclotide captured on the beads using HPLC, *see* Subheading [3.2.4](#)).

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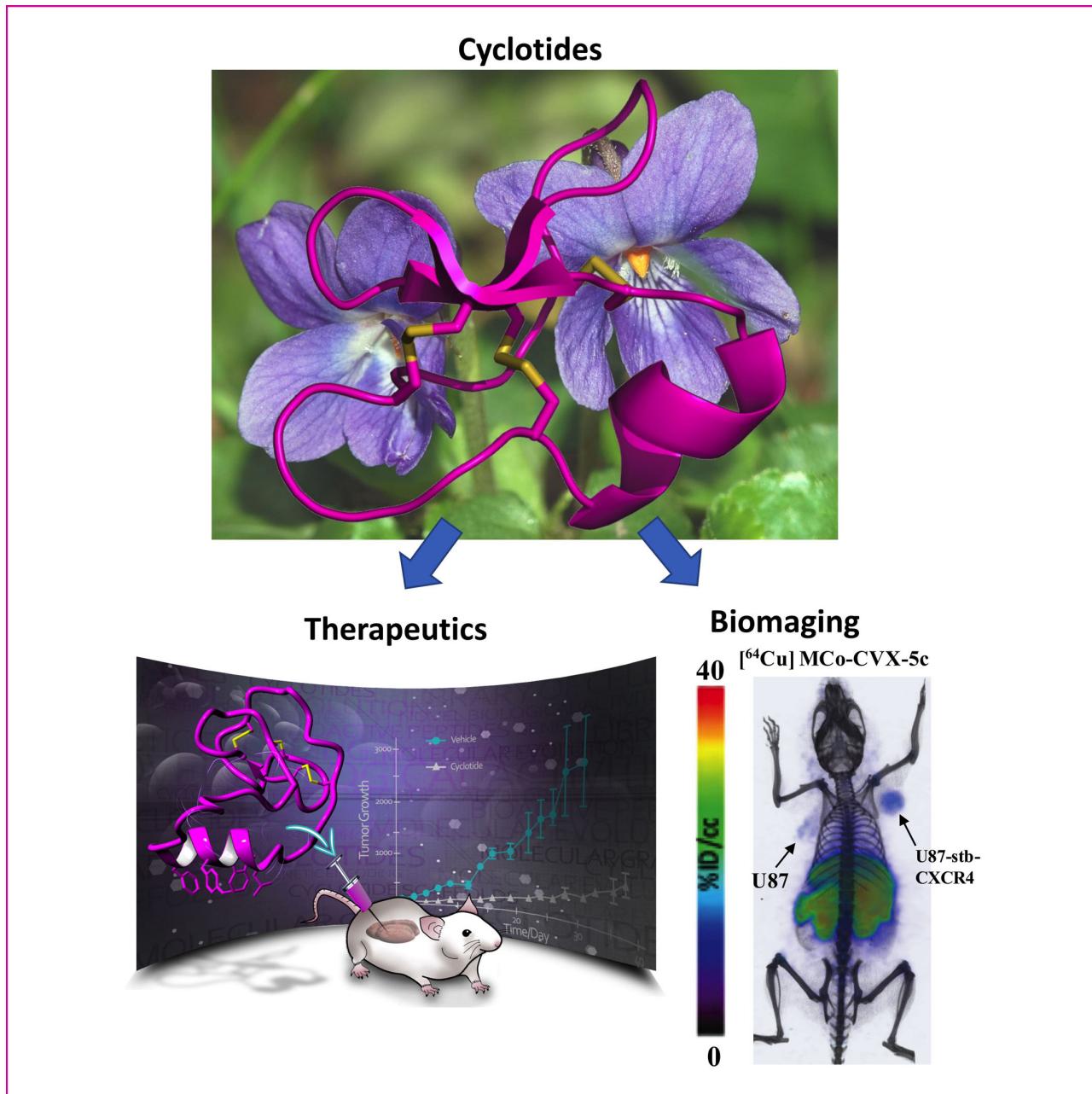
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# Cyclotides: Overview and Biotechnological Applications

Andrew Gould<sup>[a]</sup> and Julio A. Camarero\*<sup>[a, b]</sup>



Cyclotides are globular microproteins with a unique head-to-tail cyclized backbone, stabilized by three disulfide bonds forming a cystine knot. This unique circular backbone topology and knotted arrangement of three disulfide bonds makes them exceptionally stable to chemical, thermal, and biological degradation compared to other peptides of similar size. In addition, cyclotides have been shown to be highly tolerant to sequence variability, aside from the conserved residues forming

the cystine knot. Cyclotides can also cross cellular membranes and are able to modulate intracellular protein–protein interactions, both *in vitro* and *in vivo*. All of these features make cyclotides highly promising as leads or frameworks for the design of peptide-based diagnostic and therapeutic tools. This article provides an overview on cyclotides and their applications as molecular imaging agents and peptide-based therapeutics.

## Introduction

Proteins have the most dynamic and diverse role of any macromolecules in the body, including catalyzing biochemical reactions, forming receptors and channels in membranes, providing intracellular and extracellular scaffolding support, and transporting molecules within a cell or from one organ to another. It is roughly estimated that there are around 20 000 different genes in the human genome.<sup>[1]</sup> This estimate provides an immense challenge to modern medicine, as disease can result when any given protein contains mutations or other abnormalities or is present in an abnormally high or low concentration. Viewed from the perspective of therapeutics, however, this represents a tremendous opportunity in terms of providing protein–protein interactions to target and alleviate human disease.<sup>[2]</sup> Protein and peptide-based therapeutics have several advantages when targeting protein–protein interactions, due to their high specificity and selectivity.<sup>[3]</sup> However, they also show important limitations, such as limited stability to proteolysis and inability to cross biological membranes.<sup>[4]</sup> In response to this challenge, a number of technologies are starting to emerge to address these issues.<sup>[3d,5]</sup>

Special attention has been recently given to the use of highly constrained polypeptides as extremely stable and versatile scaffolds for the production of high affinity ligands for specific protein capture and/or development of therapeutics.<sup>[6]</sup> Cyclotides are fascinating microproteins ( $\approx$ 30 residues long) present in several families of plants.<sup>[7]</sup> Naturally occurring cyclotides display numerous biological properties, such as protease inhibitory, antimicrobial, insecticidal, cytotoxic, anti-HIV, and hormone-like activities.<sup>[8]</sup> They share a unique head-to-tail circular knotted topology of three disulfide bridges, with one disulfide penetrating through a macrocycle formed by the two other disulfides and interconnecting peptide backbones, forming what is called a cystine knot topology (Figure 1). Cyclotides can be considered natural combinatorial peptide libraries,

structurally constrained by the cystine knot scaffold and head-to-tail cyclization, but in which hypermutation of essentially all residues is permitted with the exception of the strictly conserved cysteines that comprise the knot.<sup>[9]</sup>

The main features of cyclotides are a remarkable stability due to the cystine knot,<sup>[10]</sup> small size (making them readily accessible to chemical synthesis),<sup>[11]</sup> recombinant expression using standard expression systems,<sup>[12]</sup> and excellent tolerance to sequence variations.<sup>[9]</sup> For example, the first cyclotide to be discovered, kalata B1, is an orally effective uterotonic,<sup>[13]</sup> and other engineered cyclotides based on kalata B1 have also been shown to be orally bioactive.<sup>[14]</sup> In addition, some cyclotides can cross mammalian cell membranes<sup>[15]</sup> and are able to target intracellular protein interactions both *in vitro* and *in vivo*.<sup>[16]</sup> Cyclotides thus appear to be promising leads or frameworks for the development of novel peptide-based diagnostics, therapeutics, and research tools.<sup>[17]</sup> This article provides a brief overview of their properties and potential use as molecular frameworks for the design of peptide-based diagnostic and therapeutic tools.

## Discovery

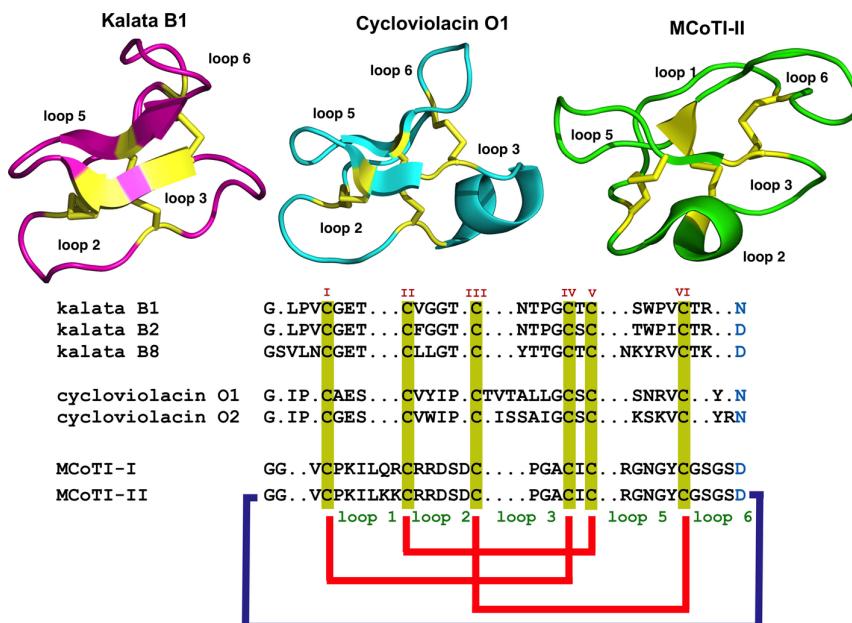
The discovery of the first cyclotide was accomplished in the late 1960s by a Norwegian doctor, Lorentz Gran,<sup>[18]</sup> who was studying an indigenous medicine in Africa that was used to accelerate childbirth. This traditional remedy was based on a medicinal herbal tea made by boiling the plant *Oldenlandia affinis* (Rubiaceae family), from which the cyclotide kalata B1 originates, in water; this shows the stability of kalata B1 at high temperatures up to 100 °C.<sup>[19]</sup> The active uterotonic component of the tea was found to be a peptide around 30 residues long that was called kalata B1. The sequence and structure of kalata B1, however, were not defined at that time, given the limitations of the protein chemistry techniques available in the early 1970s.<sup>[18]</sup> It was not until 1995 that the cystine knot backbone-cyclized nature of kalata B1 was first elucidated (Figure 1).<sup>[13]</sup>

At about the same time, different research groups discovered several other macrocyclic peptides of similar size, sequence, and structure that were isolated from plants of the Rubiaceae and Violaceae families (Figure 1).<sup>[20]</sup> This led to the definition of the cyclotide family of proteins in 1999, based on their common backbone-cyclized cystine knot topology and sequence homology.<sup>[21]</sup> Cyclotides have now been isolated from the Cucurbitaceae, Fabaceae, Solanaceae, and Apocynaceae families, in addition to Rubiaceae and Violaceae, with the

[a] Dr. A. Gould, Prof. Dr. J. A. Camarero  
Department of Pharmacology and Pharmaceutical Sciences  
University of Southern California  
Los Angeles, CA 90089-9121 (USA)  
E-mail: jcamarer@usc.edu

[b] Prof. Dr. J. A. Camarero  
Department of Chemistry, University of Southern California  
Los Angeles, CA 90089-9121 (USA)

 The ORCID identification numbers for the authors of this article can be found under <https://doi.org/10.1002/cbic.201700153>.



**Figure 1.** Primary and tertiary structures of cyclotides belonging to the Möbius (kalata B1, PDB ID: 1NB1), bracelet (cycloviolacin O1, PDB ID: 1NBJ), and trypsin inhibitor (MCoTI-II, PDB ID: 1B9) subfamilies. The sequence of kalata B8, a hybrid cyclotide isolated from the plant *O. affinis*, is also shown. Conserved Cys and Asp/Asn (required for cyclization) residues are marked in yellow and light blue, respectively. Disulfide connectivities and backbone cyclization are shown in red and by a dark blue line, respectively. Molecular graphics were created by using PyMol.

latter two comprising the majority of known cyclotides (Figure 2).<sup>[22]</sup>

Andrew Gould received his bachelor's degree in chemistry from the University of California at Irvine (UCI). He joined Dr. Camarero's research group at the University of Southern California, Los Angeles in 2010 as a graduate student, where he obtained his PhD in Pharmacology in 2016.



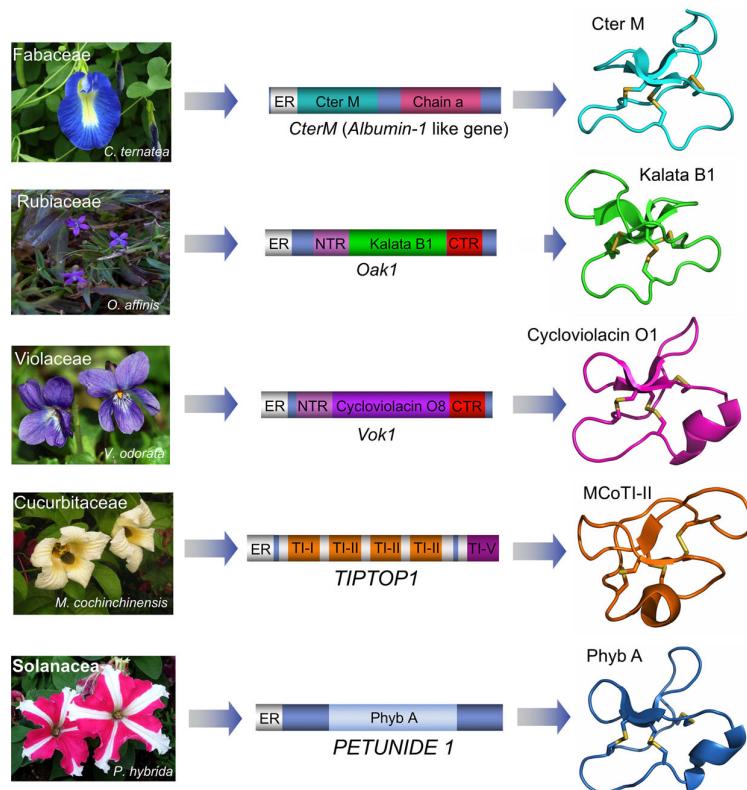
Julio A. Camarero started his studies in chemistry at the University of Barcelona (Spain), where he received his masters degree in 1992, and defended his PhD thesis in 1996. He then joined the group of Professor Tom W. Muir at The Rockefeller University as a Burroughs Wellcome Fellow. In 2000, he moved to the Lawrence Livermore National Laboratory as a Distinguished Lawrence Fellow where he became staff scientist and head of the laboratory in 2003. He joined the University of Southern California in 2007 as an associate professor and became a full professor in 2016.



Although most of the cyclotides come from the coffee and violet families, the distribution of cyclotides within these families is quite different. All of the plants studied from the violet family have been found to contain cyclotides, but only about 5% of the plants that have been analyzed from the coffee family have been shown to contain cyclotides.<sup>[22]</sup> Typically, a single plant contains multiple cyclotides ( $\approx 10$ –160), which are usually distributed across all tissues, including flowers, leaves, stems, roots, and in some cases, even seeds.<sup>[23]</sup>

Initial efforts to discover cyclotides were almost exclusively based on isolation of the cyclotides from the plant, followed by chemical characterization of the corresponding peptides.<sup>[24]</sup> The use of modern chemical approaches reduced the amount of plant material required for a full characterization of the cyclotides contained in the sample compared to previous methods. For example, MALDI-TOF/TOF and LC-MS/MS techniques have been recently used for the efficient identification of cyclotides from very small plant samples (e.g., 1 cm<sup>2</sup> of leaf tissue).<sup>[25]</sup> The recent combination of HPLC and MALDI-TOF with microwave-based extraction techniques has also been recently employed for the efficient isolation and characterization of cyclotides.<sup>[26]</sup>

The fact that cyclotides are ribosomally produced from genetically encoded protein precursors has also allowed the recent use of in silico screening approaches for the identification of cyclotides in plants.<sup>[27]</sup> For example, a recent pilot study was able to identify 145 unique cyclotide analogues from 30 different plant genomes comprising ten families.<sup>[28]</sup> In a recent study, the use of in silico transcriptomic and proteomic screening identified 164 cyclotides in *Viola tricolor*, which led to the



**Figure 2.** Genetic origin of cyclotides in plants. Rubiaceae and Violaceae plants have dedicated genes for the production of cyclotides. The genes encode protein precursors containing an ER signal peptide, an N-terminal pro-region, an N-terminal repeat (NTR), a mature cyclotide domain, and a C-terminal flanking region (CTR).<sup>[40]</sup> Cyclotides from the Fabaceae family of plants, recently isolated from *C. ternatae*, are produced from precursor proteins containing an ER signal peptide, immediately followed by the cyclotide domain, which is flanked at the C terminus by a peptide linker and the albumin A-chain. In this case, the cyclotide domain replaces the albumin-1 B-chain.<sup>[7]</sup> Cyclotides from the trypsin inhibitor subfamily are produced from TIPTOP proteins, which contain a tandem series of cyclic trypsin inhibitors terminating with an acyclic trypsin inhibitor.<sup>[38b]</sup> The protein precursors for cyclotides from the Solanaceae family are encoded in genes similar to those found in the Rubiaceae and Violaceae plants, with dedicated precursor proteins that have an ER signal, a pro-region, the linear peptide precursor, and end with a hydrophobic tail.<sup>[45a]</sup> Cyclotides structures were generated by using PyMol and PDB IDs: 2LAM (Cter M), 1NB1 (kalata B1), 1NBJ (cycloviolacin O1) and 1IB9 (MCoTi-II). The structure of cyclotide Phyb A was obtained by homology modeling with the structure of cycloviolacin O1 (PDB ID: 1NBJ) as a template.

authors of this study to estimate the total number of cyclotides in the Violaceae family alone to be around 150000.<sup>[29]</sup> In silico mining of genomes, combined with molecular biology approaches, has been used for the discovery of a large number of new ribosomal natural products, including cyclotides.<sup>[27]</sup> For example, database mining approaches were recently utilized for the identification of cyclotide-like sequences in the plant *Zea mays* (maize), which were later confirmed to be expressed at the mRNA level in the root system of the plant.<sup>[30]</sup>

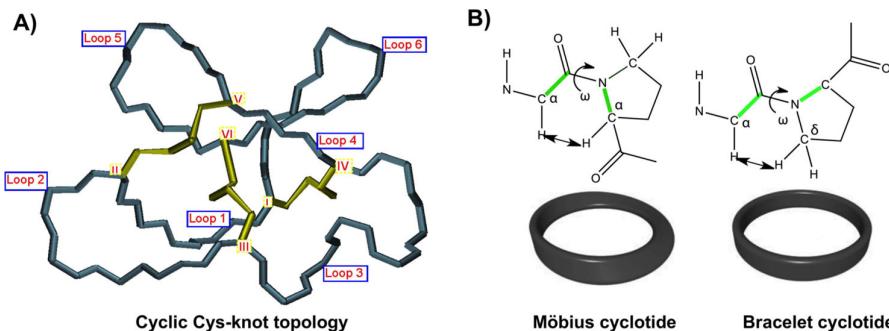
Combining the power of in silico mining of transcriptomes and/or genomes with highly efficient chemical extraction and analysis protocols using HPLC and MALDI-TOF would likely be the ultimate preferred approach for high-throughput discovery of novel cyclotide and related analogues. This approach was also recently employed for the rapid discovery of novel cyclotides.<sup>[31]</sup>

The CyBase database was recently created to allow easy access to the large number of cyclotide sequences that have been found thus far.<sup>[32]</sup> This database can be accessed through

a publicly available website (<http://CyBase.org.au>) and contains around 300 known cyclotides, as well as several useful tools for their sequence analysis. CyBase also contains sequence information on other cyclic peptides produced from ribosomally synthesized polypeptide precursors.

## Structure

All naturally occurring cyclotides range in size from 28 to 37 residues, contain six cysteine residues, and are backbone-cyclized (Figure 1). The Cys residues are oxidized to form three disulfide bonds that adopt the cyclic cystine-knot topology, that is, the disulfide bridges Cys<sup>I</sup>–Cys<sup>IV</sup> and Cys<sup>II</sup>–Cys<sup>V</sup> form a ladder arrangement, with the disulfide bridge Cys<sup>III</sup>–Cys<sup>VI</sup> running through them (Figure 3A). The interlocking nature of the cyclic cystine knot (CCK) motif makes the cyclotide backbone very compact, providing a highly rigid structure.<sup>[33]</sup> As a result, cyclotides are extremely stable compounds that are resistant to thermal and chemical denaturation and to enzymatic degradation.<sup>[17a, 34]</sup> Proof of this extraordinary stability is evident from



**Figure 3.** General structural features of the cyclic cystine knot (CCK) topology found in all cyclotides. A) Detailed 3D structure of the CCK and the connecting loops found in cyclotides. The six Cys residues are labeled I–VI, whereas loops connecting the different Cys residues are designated as loop 1–6, in numerical order from the N to the C terminus. B) Möbius cyclotides contain a *cis*-Pro residue in loop 5 that induces a local 180° backbone twist, whereas bracelet cyclotides do not.

the discovery of the first cyclotide, kalata B1, which was able to remain structurally intact and biologically active after being extracted with boiling water to make a medicinal herbal tea.<sup>[18]</sup> Most linear peptides and proteins treated under the same conditions would not retain their native structure and, consequently, would lose their biological activity.

Cyclotides are classified into three subfamilies known as the Möbius, bracelet, and trypsin inhibitor cyclotide subfamilies.<sup>[35]</sup> Although all subfamilies have the same cyclic cystine knot topology, that is, Cys disulfide connectivities, the composition of the loops is slightly different.

The Möbius subfamily of cyclotides, which includes kalata B1, contain a *cis*-proline residue at loop 5 that results in a slight twist of the backbone, whereas bracelet cyclotides do not (Figure 3B).<sup>[13]</sup> Bracelet cyclotides are slightly larger than Möbius cyclotides and are more structurally diverse. They are also much more numerous than Möbius cyclotides and make up approximately two-thirds of the known sequenced cyclotides.<sup>[36]</sup> Despite their numerical dominance, bracelet cyclotides are more difficult to fold *in vitro* than either Möbius or trypsin inhibitor cyclotides, thus making them more challenging to synthesize chemically by using standard peptide synthesis protocols. As a consequence, this type of cyclotide has been used less in the development of the biotechnological applications that will be described later in this article.

More recently, a novel suite of cyclotides possessing novel sequence features, including a lysine-rich nature, has been isolated from two species of Australasian plants from the Violaceae family.<sup>[37]</sup> These newly discovered cyclotides were found to bind to lipid membranes and were cytotoxic against cancer cell lines while showing low toxicity against red blood cells, which could be advantageous for potential therapeutic applications.

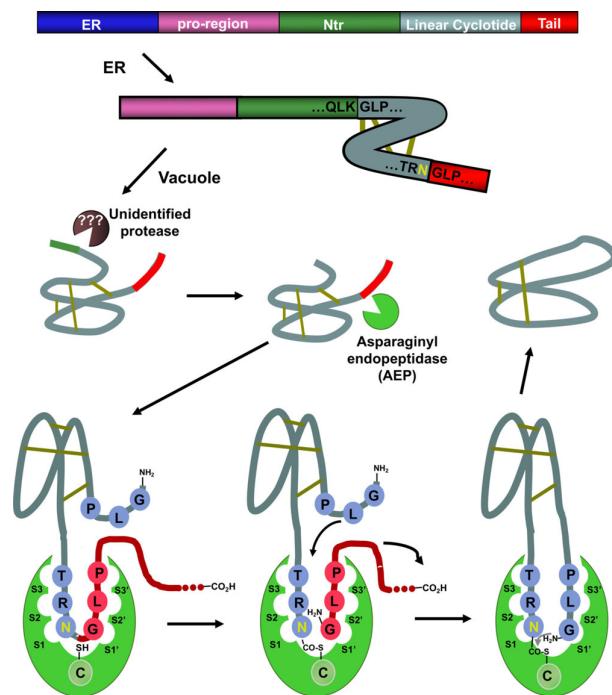
The trypsin inhibitor subfamily of cyclotides is the smallest of the three, consisting of only a small number of cyclotides isolated from the seeds of the plant *Momordica cochinchinensis* (Cucurbitaceae family)<sup>[38]</sup> that are potent trypsin inhibitors.<sup>[23a]</sup> These cyclotides do not share significant sequence homology with the other cyclotides beyond the presence of the three-cystine bridges that adopt a similar backbone-cyclic cys-

tine knot topology (Figure 1) and are more related to linear cystine knot squash trypsin inhibitors. For this reason, they are also sometimes referred as cyclic knottins.<sup>[39]</sup> Cyclotides from this family possess a longer sequence in loop 1, making the cystine knot slightly less rigid than in cyclotides from the other two subfamilies.

## Biosynthesis of Cyclotides

Cyclotides are ribosomally produced from processing of dedicated genes that, in some cases, encode multiple copies of the same cyclotide, and in others, mixtures of different cyclotide sequences.<sup>[40]</sup> The first genes encoding cyclotide precursor proteins were discovered in the plant *O. affinis* (Rubiaceae family) for the kalata cyclotides (Figure 2).<sup>[41]</sup> The gene encoding the precursor protein of cyclotide kalata B1 (Oak 1) encodes a protein containing an endoplasmic reticulum (ER)-targeting sequence, a pro-region, a highly conserved N-terminal repeat (NTR) region, a mature cyclotide domain, and a hydrophobic C-terminal tail (Figure 4).<sup>[42]</sup> Similar genes have also been found in other plants from the Violaceae family.<sup>[43]</sup> Since then, genes encoding cyclotide protein precursors have also been found in other plants from the Rubiaceae and Violaceae families.<sup>[44]</sup> More recently, cyclotide precursor genes have also been identified in plants from the Solanaceae, Fabaceae, and Cucurbitaceae families (Figure 2).<sup>[7,38b,45]</sup> These new genes provide novel protein precursor architectures, indicating high diversity in the way cyclotides are produced in nature.

The complete mechanism of how cyclotide precursors are processed and cyclized has not yet been completely elucidated (Figure 4). However, recent studies indicate that an asparaginyl endopeptidase (AEP)-like ligase is a key element in the C-terminal cleavage and cyclization of cyclotides. The transpeptidation reaction involves an acyl transfer step from the acyl-AEP intermediate to the N-terminal residue of the cyclotide domain.<sup>[46]</sup> AEPs are Cys proteases that are very common in plants and specifically cleave the peptide bond at the C terminus of asparagine and, less efficiently, aspartic acid residues. All of the cyclotide precursors identified so far contain a well-conserved Asn/Asp residue at the C terminus of the cyclotide domain in



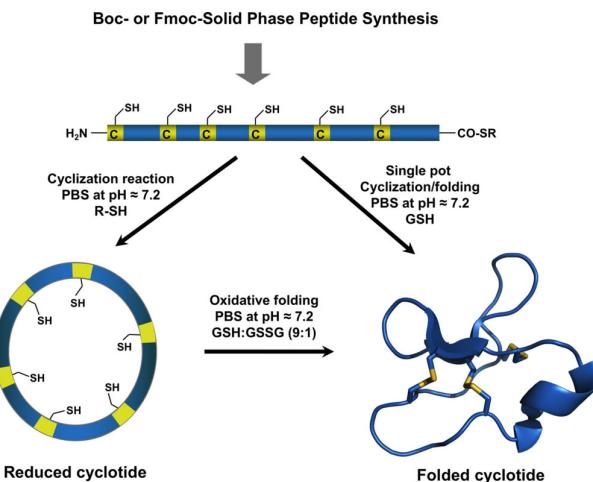
**Figure 4.** Scheme showing the major steps thought to occur during the bio-synthesis of cyclotides. The case for processing of kalata B1 is shown. It has been proposed that the cyclization step is mediated by an asparaginyl endopeptidase (AEP), a common Cys protease found in plants. The cyclization takes place at the same time as the cleavage of the C-terminal pro-peptide from the cyclotide precursor protein through a transpeptidation reaction. The transpeptidation reaction involves an acyl transfer step from the acyl-AEP intermediate to the N-terminal residue of the cyclotide domain.<sup>[40]</sup> The kalata B1 protein precursor contains an ER signal peptide, an N-terminal pro-region, an NTR, a mature cyclotide domain, and a CTR.

loop 6, which is consistent with the role of AEPs in the cleavage/cyclization step. In agreement with this, a recent study has shown that AEP-like enzymes are required for the production of mature cyclotides from *O. affinis*. The AEP-like enzyme rOaAEP1<sub>b</sub>, cloned from the *O. affinis* genome, was recombinantly produced and shown to efficiently ligate the C and N termini of an N-terminal cleaved cyclotide precursor.<sup>[47]</sup> Independently, another AEP-like ligase, called butelase-1, has recently been isolated from the cyclotide-producing plant *Clitoria ternatea*. This ligase showed efficient capabilities in cyclizing various peptides, including linear cyclotide precursors containing a C-terminal recognition sequence.<sup>[48]</sup> Despite the significant progress toward an understanding at the mechanistic level of how cyclotides are produced in plants, there still is not much known about the N-terminal cleavage process and the protease involved at that step.

## Chemical Production of Cyclotides

Cyclotides are relatively small polypeptides,  $\approx$ 30–40 amino acids long, and therefore the linear precursors can be readily synthesized by chemical methods using solid-phase peptide synthesis (SPPS).<sup>[49]</sup> Backbone cyclization of the corresponding

linear precursor can be readily accomplished in aqueous buffers under physiological conditions by using an intramolecular version of native chemical ligation (NCL)<sup>[50]</sup> on linear precursors containing an N-terminal cysteine and an  $\alpha$ -thioester group at the C terminus (Figure 5).<sup>[51]</sup> Peptide  $\alpha$ -thioesters can be easily



**Figure 5.** Chemical synthesis of cyclotides by means of an intramolecular native chemical ligation (NCL). This approach requires the chemical synthesis of a linear precursor bearing an N-terminal Cys residue and an  $\alpha$ -thioester moiety at the C terminus. The linear precursor can first be cyclized under reductive conditions and then folded by using a redox buffer containing reduced and oxidized glutathione (GSH).<sup>[51]</sup> Alternatively, the cyclization and folding can be efficiently accomplished in a single pot reaction when the cyclization is carried out in the presence of reduced GSH as the thiol co-factor.<sup>[11]</sup>

generated by standard SPPS approaches using either Boc- or Fmoc-based chemistry.<sup>[11]</sup> Once the peptide is cleaved from the resin, the linear cyclotide precursors can be cyclized and folded sequentially. More recently, it has been shown that the cyclization and folding can be carried out in a single pot reaction by using glutathione (GSH) as a thiol additive.<sup>[52]</sup> This approach has been successfully used to chemically generate many native and engineered cyclotides (for a recent review of this topic, see ref. [11]).

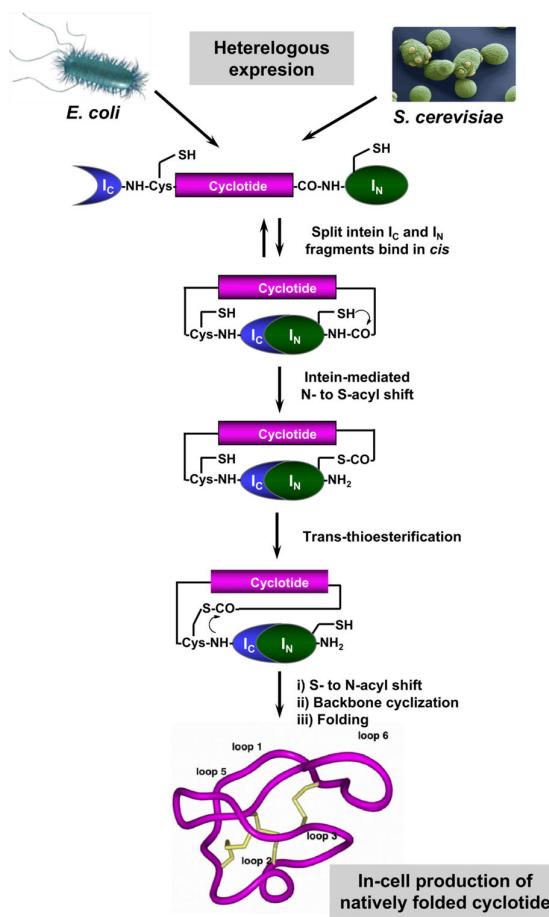
Cyclotides can also be produced by chemoenzymatic cyclization of the corresponding synthetic linear precursors using AEP-like ligases, as mentioned earlier.<sup>[47, 48]</sup> These enzymes do not require the cyclotide linear precursor to be natively folded for the cyclization step to proceed efficiently.<sup>[48]</sup> The serine protease trypsin has also been used to produce several cyclotides, based on the naturally occurring trypsin inhibitor cyclotide MCoTI-II.<sup>[53]</sup> In this work, folded linear cyclotide precursors bearing the P1 and P1' residues at the C and N termini, respectively, were used as a viable substrate for trypsin-mediated cyclization, thus enabling synthesis of the cyclic backbone without the need for a C-terminal  $\alpha$ -thioester. The use of trypsin-mediated cyclization provides a very efficient route for obtaining cyclotides with trypsin inhibitory properties. It should be noted, however, that although the cyclization yield can be extremely efficient ( $\approx$ 92% for naturally occurring cyclotide MCoTI-II), the

introduction of mutations that affect binding to the proteolytic enzyme could affect the cyclization yield.<sup>[9a]</sup>

Transpeptidases like sortase A (SrtA) can also be used for the production of cyclotides from synthetic linear precursors.<sup>[54]</sup> It is worth noting, however, that due to the sequence requirements for SrtA, the heptapeptide motif (LPVTGGG) remains at the ligation site, and this should be considered when producing biologically active cyclotides.

## Recombinant Expression of Cyclotides

Recent advances in the fields of protein engineering and molecular biology make the biological synthesis of backbone-cyclized polypeptides possible by using standard heterologous expression systems (for a recent review on this topic, see



**Figure 6.** Heterologous expression of cyclotides by protein trans-splicing (PTS).<sup>[57–58]</sup> In this approach, the linear cyclotide precursor is fused in-frame at the C and N termini directly to the  $I_N$  and  $I_C$  polypeptides of the *Npu* DnaE split intein. None of the additional native C- or N-extein residues were added in this construct. In this work, the native Cys residue located at the beginning of loop 6 of MCoT-I was used to facilitate backbone cyclization. The N and C termini of the linear cyclotide precursor were linked together through a native peptide bond through a transpeptidation reaction mediated by the self-processing domains of the split intein. This approach has been successfully used for the production of bioactive cyclotides in eukaryotic and prokaryotic expression systems.<sup>[57–58]</sup>

ref. [11]). The discovery of intein-mediated protein splicing both in *cis* and *trans* has made possible the generation of backbone-cyclized polypeptides by using standard expression systems (Figure 6). Our group pioneered the use of intein-mediated backbone cyclization for the biosynthesis of fully folded cyclotides inside bacterial cells by using heterologous expression systems.<sup>[9a,55]</sup> This initial approach used an intramolecular version of intein-mediated ligation (also called expressed protein ligation (EPL)).<sup>[12]</sup> This cyclization method has also been successfully used for the recombinant expression of other naturally occurring disulfide-rich backbone-cyclized polypeptides such as the Bowman–Birk inhibitor SFTI-1 and several  $\theta$ -defensins.<sup>[9a,56]</sup>

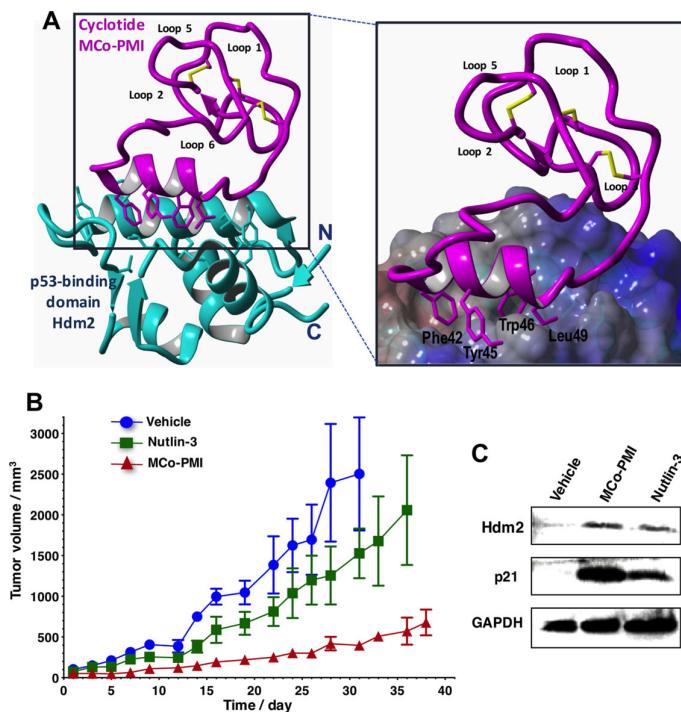
Backbone cyclization by intein-mediated protein trans-splicing (PTS) has also been reported for the efficient production of naturally occurring and engineered cyclotides in prokaryotic and eukaryotic expression systems (Figure 6).<sup>[57]</sup> In-cell expression of folded cyclotides is quite efficient at being able to provide intracellular concentrations in the range of 20–40  $\mu\text{M}$ , which are appropriate for performing in-cell screening.<sup>[57]</sup> This level of expression in *Escherichia coli* equals  $\approx 10$  mg of folded cyclotide per 100 g of wet cells.<sup>[58]</sup> More importantly, in-cell production makes possible the generation of large genetically encoded libraries of cyclotides inside live cells that can be rapidly screened for the selection of novel sequences able to modulate or inhibit the biological activities of particular biomolecular targets.<sup>[57b]</sup>

The recombinant production of cyclotides facilitates the production of cyclotides labeled with NMR-active isotopes such as  $^{15}\text{N}$  and/or  $^{13}\text{C}$  in a very inexpensive fashion.<sup>[33,59]</sup> Having access to  $^{15}\text{N}$ - and/or  $^{13}\text{C}$ -labeled cyclotides facilitates the use of heteronuclear NMR spectroscopy to study structure–activity relationships (SAR) of any biologically active cyclotides and their molecular targets. This was recently demonstrated in the structural studies carried out on a cyclotide engineered to bind the p53 binding domain of the E3 ligases Hdm2 and HdmX (Figure 7). PTS has also been successfully used in the production of other Cys-rich backbone-cyclized polypeptides.<sup>[60]</sup>

## Biological Activities of Naturally Occurring Cyclotides

The biological function of the naturally occurring cyclotides of the Möbius and bracelet subfamilies in plants seems to be primarily as host–defense agents, as deduced from their activity against insects.<sup>[7,41,61]</sup> Cyclotides have been shown to efficiently inhibit the growth and development of nematodes and trematodes<sup>[62]</sup> and of mollusks.<sup>[63]</sup>

Cyclotides seem to exert their biological activity by interacting with cellular membranes and disrupting their normal function. For example, the midgut membranes of Lepidopteran species are severely disrupted after ingesting cyclotides.<sup>[64]</sup> The molecular mechanism of how cyclotides disrupt cellular membranes has been widely studied, and at least for cyclotide kala-ta B1, it is well established that the first step involves the specific binding of the cyclotide to the phosphatidylethanolamine phospholipids present in the cellular membrane.<sup>[65]</sup> This initial



**Figure 7.** Structure and in vivo activity of the first cyclotide designed to antagonize an intracellular protein–protein interaction in vivo.<sup>[16]</sup> A) Solution structure of an engineered cyclotide MCo-PMI (magenta) and its intracellular molecular target, the p53 binding domain of oncogene Hdm2 (blue). The cyclotide binds with low-nanomolar affinity to the p53-binding domains of both Hdm2 and HdmX. The overexpression of these two proteins, Hdm2 and HdmX, is a common mechanism used by many tumor cells to inactivate the p53 tumor suppressor pathway, promoting cell survival. Targeting Hdm2 and HdmX has emerged as a validated therapeutic strategy for treating cancers with wild-type p53. B) Cyclotide MCo-PMI activates the p53 tumor suppressor pathway and blocks tumor growth in a human colorectal carcinoma xenograft mouse model. HCT116 p53<sup>+/+</sup> xenografts mice were treated with vehicle (5% dextrose in water), nutlin 3 (10 mg kg<sup>-1</sup>), or cyclotide (40 mg kg<sup>-1</sup>, 7.6 mmol kg<sup>-1</sup>) by intravenous injection daily for up to 38 days. Tumor volume was monitored by caliper measurement. C) Tumor samples were also subjected to SDS-PAGE and analyzed by western blotting for p53, Hdm2, and p21, indicating activation of p53 in tumor tissue.

binding step causes internalization of the cyclotide into the membrane, compromising its physical integrity and triggering the formation of pores and/or leakage of cell contents.<sup>[66]</sup> Aside from their insecticidal and nematocidal activities, cyclotides have also been shown to have potential pharmacologically relevant activities, which include antimicrobial and antitumor activities.

Cyclotides from the Möbius and bracelet subfamilies have hydrophobic and hydrophilic patches located in different regions of their surface, resembling to some extent the amphipathic character of classical antimicrobial peptides. For example, the cyclotide kalata B1 has been found to have antimicrobial activity against Gram-negative and Gram-positive bacteria.<sup>[67]</sup> Similar antibacterial activities have been found in cyclotides isolated from *Hedyotis biflora* (Rubiaceae family)<sup>[68]</sup> and *C. ternatae* (Fabaceae family).<sup>[45b]</sup> The most active antimicrobial cyclotide tested so far seems to be the bracelet cyclotide cycloviolacin O2,<sup>[69]</sup> which has been shown to have antimicrobial activity against *Staphylococcus aureus* in a mouse infection

model.<sup>[70]</sup> It is worth noting, however, that the anti-microbial activity of cyclotides when tested in vitro seems to occur only under non-physiological conditions involving the use of low ionic strength buffers, which seriously limits its potential for the design of antimicrobial therapeutics.

The anti-HIV activity of cyclotides has been one of the most extensively studied so far, due to its potential pharmacological applications.<sup>[20a, 71]</sup> Gustafson and coworkers reported the first cyclotides with anti-HIV activity as part of a screening program to identify novel natural antiviral compounds.<sup>[71a, 72]</sup> More recently, several other cyclotides from the bracelet and Möbius subfamilies were also shown to have anti-HIV activity.<sup>[71c, d]</sup>

Although the exact molecular mechanism of action is not fully understood, the inability of cyclotides to inhibit HIV reverse transcriptase activity seems to suggest that the antiviral activity occurs before entry of the virus into the host cell.<sup>[20a]</sup> Recent studies have also shown a correlation between the hydrophobic character of cyclotides and their anti-HIV activities.<sup>[71d, 73]</sup> The fact that cyclotides can bind phospholipids present in the cellular membrane might suggest that the probable mode of anti-HIV activity could happen through a mechanism that affects the binding and/or fusion of the virus to the cellular membrane. However, it is unclear whether the cyclotide activity could be the result of binding to the host cell membrane, the viral envelope, or both.

Several cyclotides have been reported to have selective cytotoxicity against several cancer cell lines, including primary cancer cell lines, when compared to normal cells.<sup>[74]</sup> More recently, the cytotoxic activity of cyclotide vingo 5 from *Viola ignobilis* has been shown to be apoptosis-dependent when tested in HeLa cells.<sup>[75]</sup> In addition, three new cyclotides isolated from *Hedyotis diffusa*, a Chinese medicinal plant from the Rubiaceae family, have been shown to induce apoptosis and inhibit proliferation and migration of several prostate cancer cell lines.<sup>[76]</sup> Cyclotide DC3, the most active of the three, was able to inhibit tumor growth in a mouse xenograft model. Similar cytotoxic activities have been also reported in vitro against MCF-7 (breast carcinoma) and Caco-2 (colorectal adenocarcinoma) cells with acyclic cyclotides isolated from the plant *Palicourea rigida*.<sup>[77]</sup> Overall, the therapeutic index (i.e., the ratio between the dose required for therapeutic effects versus toxic effects on normal cells) of cytotoxic cyclotides is not very high, and therefore will require optimization before these compounds can be developed into effective anticancer agents.

More recently, the molecular targets of labor-accelerating cyclotide kalata B7 and engineered analogues were found to be the G protein-coupled oxytocin and vasopressin V<sub>1a</sub> receptors.<sup>[78]</sup>

**Table 1.** Summary of work published in engineered cyclotides and linear cyclotides/knottins with novel biological activities, leading to therapeutic and bioimaging applications.

Cyclotide modified	Biological activity	Loop	Application	Ref.
<b>Möbius subfamily</b>				
kalata B1	VEGF-A antagonist	2, 3, 5, 6	anti-angiogenic, potential anticancer activity	[79a]
	Dengue NS2B-NS3 protease inhibitor	2, 5	antiviral for Dengue virus infections	[98]
	bradykinin B1 receptor antagonist	6	chronic and inflammatory pain	[14]
	melanocortin 4 receptor agonist	6	obesity	[80]
	neuropilin-1/2 antagonist	5, 6	inhibition of endothelial cell migration and angiogenesis	[99]
	immunomodulator	5, 6	protecting against multiple sclerosis	[87]
	immunomodulator	4	protecting against multiple sclerosis	[100]
<b>Trypsin inhibitor subfamily</b>				
MCoTI-I	CXCR4 antagonist	6	antimetastatic and anti-HIV	[52, 81]
	p53-Hdm2/HdmX antagonist	6	antitumor by activation of p53 pathway	[16]
	$\alpha$ -synuclein-induced cytotoxicity inhibitor	6	Parkinson's disease-validation of phenotypic screening of genetically encoded cyclotide libraries	[57b]
MCoTI-II	MAS1 receptor agonist	6	lung cancer and myocardial infarction	[105]
	FMDV 3C protease inhibitor	1	antiviral for foot-and-mouth disease	[53]
	$\beta$ -tryptase inhibitor	3, 5, 6	inflammation disorders	[85]
	$\beta$ -tryptase inhibitor and human elastase inhibitor	1	inflammation disorders	[79b]
	CTLA-4 antagonist	1, 3, 6	immunotherapy for cancer	[101]
	tryptase inhibitor	1	anticancer	[102]
	VEGF receptor agonist	6	wound healing and cardiovascular damage	[103]
	BCR-Abl kinase inhibitor	1, 6	chronic myeloid leukemia-attempt to graft both a cell-penetrating peptide and kinase inhibitor	[104]
	SET antagonist	6	potential anticancer	[88]
	FXIIa and FXa inhibitors	1, 6	antithrombotic and cardiovascular disease	[106]
	thrombospondin-1 (TSP-1) agonist	6	microvascular endothelial cell migration inhibition and anti-angiogenesis	[107]
	antiangiogenic	5, 6	anticancer	[108]
<b>Linear trypsin inhibitor (acyclic cyclotide/linear knottin) subfamily</b>				
EETI	integrin-binding knottin conjugated with contrast microbubbles	1 <sup>[a]</sup>	ultrasound imaging of tumor angiogenesis	[93]
	fluorescence-labeled integrin-binding knottin	1 <sup>[a]</sup>	PET imaging-localizes in mouse medulloblastoma	[92]
	<sup>64</sup> Cu-labeled integrin-binding knottin	1, 5 <sup>[a]</sup>	PET probe for atherosclerosis imaging	[94]
	<sup>18</sup> F-labeled integrin-binding knottin imaging	1, 5 <sup>[a]</sup>	PET probe for angiogenesis	[94]
	<sup>99</sup> Tc-labeled integrin-binding knottin	1, 3, 5 <sup>[a]</sup>	SPECT agent for imaging integrin $\alpha_v\beta_3$	[95]
	<sup>177</sup> Lu-labeled integrin-binding knottin of integrin-positive tumors	1 <sup>[a]</sup>	SPECT agent for radionuclide therapy	[95]

[a] Loop numbering in EETI is based on sequence homology to the cyclotide MCoTI-I.

## Engineered Cyclotides with New Biological Activities

The unique properties associated with the cyclotide scaffold make them extremely valuable in the development of novel peptide-based therapeutics (Table 1).<sup>[17]</sup> As mentioned earlier, the CCK framework provides cyclotides with a compact and a highly rigid structure that gives them an exceptional resistance to chemical, physical, and biological degradation. The cyclotide scaffold also shows very high tolerance to mutations, making this an ideal molecular framework for molecular grafting and evolution for the generation of novel cyclotides with new biological activities. In addition, cyclotides from the trypsin inhibitor subfamily are not toxic to mammalian cells at concentrations up to 100  $\mu$ M<sup>[16]</sup> and have been shown to be able to cross cellular membranes<sup>[15]</sup> to target intracellular cytosolic protein-protein interactions.<sup>[16]</sup>

The pharmacologic potential of grafted cyclotides was first demonstrated in two early studies aimed at developing novel anticancer<sup>[79]</sup> and antiviral peptide-based therapeutics.<sup>[53]</sup> Tumor growth is usually associated with unregulated angiogenesis; therefore, molecules with anti-angiogenic activity have potential applications in cancer treatment. The molecular grafting of an Arg-rich peptide antagonist for the interaction of vascular endothelial growth factor A (VEGF-A) and its receptor into several loops of cyclotide kalata B1 yielded cyclotides with anti-VEGF activity.<sup>[79a]</sup> The cyclotide grafted into loop 3 showed the highest activity in blocking VEGF-A receptor binding ( $IC_{50} \approx 12 \mu$ M). Although this is the first example of a successful functional redesign of a cyclotide, it should be noted that the biological activity would still need to be improved by several orders of magnitude for a potential pharmacological application *in vivo*. A similar approach has been used more recently for targeting the bradykinin and melanocortin 4 receptors for pain and obesity management, respectively.<sup>[14, 80]</sup> It is

worth noting that the kalata B1-based bradykinin antagonist was shown to be orally bioavailable,<sup>[14]</sup> highlighting the potential of the cyclotide scaffold for the development of orally bioavailable peptide-based therapeutics. The cyclotide MCoTI-I has been recently used for the design of a potent (low nanomolar) CXCR4 antagonist.<sup>[81]</sup> The cytokine receptor CXCR4 has been associated with multiple types of cancers, where its over-expression/activation promotes metastasis, angiogenesis, and tumor growth and/or survival.<sup>[82]</sup>

Proteases are well-recognized drug targets, as they are involved in human diseases including microbial/viral infectivity.<sup>[83]</sup> In addition, many human diseases, including inflammatory and pulmonary diseases, cancer, cardiovascular, and neurodegenerative conditions have been associated with abnormal expression levels of proteases.<sup>[84]</sup> The trypsin inhibitor subfamily of cyclotides has been used for the design for protease inhibitors with pharmacological relevance. For example, a mutated version of cyclotide MCoTI-II was transformed into a potent and selective foot-and-mouth disease (FMDV) 3C protease inhibitor.<sup>[53]</sup> The same scaffold was also used in the development of  $\beta$ -tryptase and human leukocyte elastase inhibitors with low nanomolar  $K_i$  values.<sup>[79b, 85]</sup> These proteases are validated targets for inflammatory disorders.

In a recent work, a point-mutated cyclotide, kalata B1 T20K, was reported to have oral activity in a mouse model of multiple sclerosis.<sup>[86]</sup> The potential of grafted cyclotides in the context of multiple sclerosis has been also explored by grafting peptide sequences from the MOG35-55 epitope onto the cyclotide kalata B1.<sup>[87]</sup>

One of the most exciting features of the cyclotide scaffold is that some cyclotides, in particular those from the trypsin inhibitor subfamily, are able to penetrate cells. This exciting finding makes possible the delivery of biologically active cyclotides by using grafted MCoTI-based cyclotides to target intracellular protein–protein interactions. For example, we recently used cyclotide MCoTI-I to produce a potent inhibitor of the interaction between p53 and the proteins Hdm2/HdmX (Figure 7).<sup>[16]</sup> The resulting cyclotide, MCo-PMI, was able to bind with low nanomolar affinity to both Hdm2 and HdmX, showed high stability in human serum, and was cytotoxic to wild-type p53 cancer cell lines by activating the p53 tumor suppressor pathway both in vitro and in vivo (Figure 7).<sup>[16]</sup> This work constitutes the first example in which an engineered cyclotide was able to target an intracellular protein–protein interaction in an animal model of human colon carcinoma, highlighting the therapeutic potential of MCoTI cyclotides for targeting intracellular protein–protein interactions. A similar approach, but instead employing cyclotide MCoTI-II, was also used to produce a grafted cyclotide able to antagonize the SET protein, which is over-expressed in some human cancers.<sup>[88]</sup>

We have recently reported an MCoTI-grafted cyclotide (MCoCP4) that was able to inhibit  $\alpha$ -synuclein-induced cytotoxicity in yeast *Saccharomyces cerevisiae*.<sup>[57b]</sup> This was accomplished by grafting the sequence of cyclic peptide CP4 (cyclotides CLATWAVG), which was recently shown to reduce  $\alpha$ -synuclein-induced cytotoxicity in a yeast,<sup>[89]</sup> into the loop 6 of MCoTI-I.  $\alpha$ -Synuclein is a small lipid-binding protein that is prone to mis-

folding and aggregation and has been linked to Parkinson's disease by genetic evidence and its abundance in the Parkinson's disease-associated intracellular aggregates known as Lewy bodies; therefore, it is a validated therapeutic target for Parkinson's disease.

Given the good *in vivo* biological activity of MCoTI cyclotides, the biodistribution and potential of these cyclotides to cross the blood–brain barrier have recently been studied.<sup>[90]</sup> In this work, it was confirmed that cyclotide MCoTI-II is distributed predominantly to the serum and kidneys, thus confirming that it is stable in serum and suggesting that it is eliminated from the blood through renal clearance. In addition, this work also showed that, although MCoTI cyclotides have cell-penetrating properties and can modulate intracellular protein/protein interactions, cyclotide MCoTI-II showed no significant uptake into the brain.

## Screening of Cyclotide-Based Libraries

The ability to produce natively folded cyclotides *in vivo*<sup>[55b, 57b, 91]</sup> discussed previously opens the intriguing possibility of generating large libraries of genetically encoded cyclotides, potentially containing billions of members. This tremendous molecular diversity should allow the selection of strategies that mimic the evolutionary processes found in nature to select novel cyclotide sequences able to target specific molecular targets. As a proof of principle, we used this approach for the production of a genetically encoded library of MCoTI-I-based cyclotides. The library was designed to mutate every single amino acid in loops 1–5 to explore the effects on folding and trypsin binding activity of the resulting mutants.<sup>[55b]</sup> Interestingly, only two mutations (G27P and I22G) out of the 26 substitutions studied were able to negatively affect the folding of the resulting cyclotides. Although these two mutants were not able to fold efficiently, their natively folded form was still able to bind trypsin. The rest of the mutants were able to cyclize and fold with similar yields to that of the wild-type cyclotide, emphasizing the high plasticity and sequence tolerance of MCoTI-based cyclotides.<sup>[55b]</sup>

More recently, we have shown that cyclotide-based libraries can be also used for phenotypic screening in eukaryotic cells.<sup>[57b]</sup> In this work, an engineered cyclotide (MCoCP4) that was designed to reduce toxicity of human  $\alpha$ -synuclein in live yeast cells was selected by phenotypic screening from cells transformed with a mixture of plasmids encoding MCoCP4 and inactive cyclotide MCoTI-I in a ratio of 1:50000. These exciting results demonstrate the potential to perform phenotypic screening of genetically encoded cyclotide-based libraries in eukaryotic cells for the rapid selection of novel bioactive cyclotides. In addition, expression in eukaryotic systems should allow the production of cyclotides with different post-translational modifications not available in bacterial expression systems.

The recent development of efficient approaches for the chemical synthesis, cyclization, and folding of cyclotide-based libraries has allowed, for the first time, high-throughput screening on chemically generated libraries of cyclotides.<sup>[52]</sup> We have

recently demonstrated that bioactive folded MCoTI-based cyclotides can be efficiently produced in parallel by using a “teabag” approach in combination with highly efficient cyclization–folding protocols.<sup>[52]</sup> The approach described in this work also includes an efficient purification procedure to rapidly remove unfolded or partially folded cyclotides from the cyclization–folding crude. This procedure can easily be used in parallel for the purification of individual compounds but also, and more importantly, for the purification of cyclotide mixtures, therefore making it compatible with the synthesis of amino acid and positional scanning libraries to perform efficient screening of large chemically generated libraries. A similar approach was recently used to produce a potent anthrax lethal factor protease inhibitor by using the Cys-rich backbone-cyclized  $\theta$ -defensin RTD-1.<sup>[60b]</sup>

### Cyclotides as Molecular Imaging Probes

The development of adequate diagnostic molecular tools is key for early detection and monitoring in the successful treatment of many diseases, including cancer.<sup>[5]</sup> Over the past two decades, technological advances in imaging instrumentation have dramatically increased the capabilities for bioimaging, fueling the need to develop improved molecular imaging agents. Ideally, improved imaging agents should provide high affinity and selectivity for the corresponding molecular marker and greater stability. To achieve optimal contrast between healthy and diseased tissues, molecular agents should have affinities in the low-nanomolar to picomolar range, high selectivity over healthy tissue, rapid clearance from healthy tissue to reduce background signal, and high chemical and biological stability.

Properly functionalized engineered linear squash trypsin inhibitors, which share sequence homology and structure with the trypsin inhibitor subfamily of cyclotides but are not backbone-cyclized, have been shown to be excellent bioimaging and detection tools in cancer (recently reviewed in ref. [5]). Integrin-binding variants based on the *Ecballium elaterium* trypsin inhibitor II (EETI) have been extensively used as bioimaging agents (Table 1). They have been shown to provide significant tumor accumulation and low imaging signals in kidney, liver, and other organs. For example, an Alexa-Fluor 680 dye-conjugated integrin-targeting EETI variant has been shown to localize to intracranial medulloblastoma in mice after intravenous injection.<sup>[92]</sup> An integrin-targeting EETI variant conjugated to a contrast-enhanced ultrasound imaging agent has been also used for magnetic resonance imaging (MRI).<sup>[93]</sup> Different integrin-binding EETI variants labeled with  $^{18}\text{F}$  and  $^{64}\text{Cu}$  radionuclides have been used for positron emission tomography (PET) imaging,<sup>[94]</sup> whereas  $^{99}\text{Tc}$  and  $^{177}\text{Lu}$  were used for single-photon emission computed tomography (SPECT).<sup>[95]</sup>

There are still no published reports of using cyclotides as imaging agents. However, given the similar characteristics in sequence and structure (except for the head-to-tail cyclization) of the trypsin inhibitor cyclotides and the linear squash trypsin inhibitors described above, it is quite likely that this subfamily of cyclotides could be used for the development of excellent

imaging reagents. Cyclotides from this family are not toxic to mammalian cells, do not interact with membranes, and can be easily engineered to introduce novel biological functions. For example, our group has recently designed a cyclotide by using the trypsin inhibitor cyclotide MCoTI-I that was able to antagonize the cytokine G protein-coupled receptor CXCR4 with low-nanomolar affinity.<sup>[81]</sup> Overexpression of the CXCR4 in cancer cells is often correlated with a propensity for metastasis and poor prognosis,<sup>[96]</sup> and it has been proposed as a molecular biomarker for the development of diagnostic agents for therapeutic guidance and monitoring of cancer metastasis.<sup>[97]</sup> Hence, the development of cyclotide-based specific CXCR4 imaging agents should be feasible.

### Concluding Remarks

Cyclotides are becoming a well-studied family of microproteins that, given their unique properties, are also starting to gain acceptance as molecular scaffolds for the potential design of novel peptide-based therapeutics. Their unique circular backbone topology and knotted arrangement of three disulfide bonds provides a compact, highly rigid structure that confers exceptional resistance to thermal/chemical denaturation and enzymatic degradation. Cyclotides have been shown to be able to cross human cell membranes and to efficiently target protein–protein interactions *in vitro* but also, and more importantly, in animal models. The fact that cyclotides can target intracellular targets *in vivo* highlights the high stability of the cystine knot to be degraded/oxidized under complex biological conditions. The relatively small size of cyclotides also makes them readily available by chemical synthesis, allowing introduction of chemical modifications such as unnatural amino acids and PEGylation to improve their pharmacological properties. Cyclotides can also be expressed in several heterologous expression systems and are amenable to substantial sequence variation, making them ideal substrates for molecular evolution strategies to enable generation and selection of compounds with optimal binding and inhibitory characteristics. Together, these unique polypeptide characteristics make them promising leads or frameworks for peptide drug design.

Although no cyclotides have reached human clinical trials yet, the results obtained with several bioactive cyclotides in animal models hint that this might occur in a the not-too-distant future. One the main challenges that affect cyclotides, if they want to compete with small molecule therapeutics, is their oral bioavailability. Some cyclotides have been shown to be orally active but little information is available about their oral bioavailability. It is anticipated that more studies on the biopharmaceutical properties of these interesting microproteins will be available in the coming years.

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## Conflict of Interest

The authors declare no conflict of interest.

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## REVIEWS

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**Tied in knots:** Cyclotides are globular microproteins with a unique head-to-tail cyclized backbone, stabilized by three disulfide bonds forming a cystine knot. The present review provides an overview on cyclotides and their applications as molecular imaging agents and peptide-based therapeutics.



*A. Gould, J. A. Camarero\**

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**Cyclotides: Overview and Biotechnological Applications**